**University of Alberta**

# Molecular Interactions Between MHC B and its Co-chaperone, UNC-45, in *C. elegans*

by

Laura Horlick ©

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# Abstract

UNC-45 is an essential myosin co-chaperone that was first characterized in the nematode *Caenorhabditis elegans*. I examined two aspects of UNC-45 function (i) which region of one of the two body wall muscle myosins, MHC B, is required to localize UNC-45 to the thick filament; and (ii) is the localization of UNC-45 to the thick filament independent of its function. Using chimeric myosins, I found that a region of the MHC B rod, and not the head as previously thought, correctly localizes UNC-45 to the thick filament. I also examined whether the chimeric myosins could properly assemble in the absence of UNC-45. Myosin containing the MHC B head required UNC-45 to assemble whereas myosin containing the head of a second isoform, MHC A, did not require UNC-45. This suggests that MHC A and MHC B differ in their requirements for chaperone activities of UNC-45.

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## Abbreviations used in this thesis

BSA: bovine serum albumun

CNBr: Cyanogen bromide

DTT: dithiothreitol

GC UNC-45: general cell UNC-45

GFP: green fluorescent protein

GST: glutathione-S-transferase

HA: hemaglutinin

IPTG: isopropyl-β–D-thiogalactopyranoside

MHC: myosin heavy chain

MO: morpholino oligonucleotide

Pat: paralyzed and arrested at two-fold stage

PBS: phosphate buffered saline

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PIPES: 1, 4 piperazinediethanesulfonic acid

SM UNC-45: striated muscle UNC-45

TPR: tetratricopeptide repeat

UNC: uncoordinated

UCS: UNC-45/CRO1/She4p

*(0)*: null mutation

*(ts)*: temperature sensitive mutation

(d): dominant negative mutation

# 1. Introduction

With the scientific advances made in today's society, it is becoming possible to understand the mechanics of how our bodies function as well as the underlying genetic problems involved when the body does not function properly. For example, there are multiple causes of myopathies known to occur in humans where there is weakness and atrophy in muscle systems ranging from skeletal to heart. This type of disease affects many people, and because the underlying genetic defects remain unknown, few recover. Recently, it has been shown that many myopathies are caused by mutations in proteins involved in the sarcomere, the contractile unit of muscle (Bonnemann and Laing, 2004). For example, a mutation in the head of myosin heavy chain IIa (MyHC IIa), the major molecular motor involved in muscle contraction, has been implicated in causing familial congenital myopathy (Oldfors et al., 2004). To fully understand how a single mutation in a myosin gene has such huge implications in muscle function, the entire process must be broken down and examined on a molecular and genetic scale.

Although the goal is to understand how the human system functions, it is difficult to perform the appropriate analyses and experiments on a human or other mammalian system. Few progeny are produced; embryos develop internally; mothers are opaque, preventing visualization of tissues during embryo and fetal development; and generation times are very long. Since the majority of genes and developmental pathways are conserved throughout evolution, researchers can use much smaller, more amenable model organisms to help understand how human systems function. For example: how a mutation in a myosin gene in skeletal muscles may cause a myopathy and how can it be overcome.

This thesis describes my use of the model organism *Caenorhabditis elegans,* a tiny soil nematode, to further our understanding of the how a myosin heavy chain gene (MHC B) and its co-chaperone, UNC-45, interact to form the thick filaments in the body wall muscle sarcomeres.

### 1.1. *C. elegans* as a model for studying muscle systems

Since its introduction over four decades ago, *C. elegans* has proven to be an excellent model system for genetic and developmental analysis of many processes. Its small size coupled with its short reproduction time and transparent body (which allows for visualization of internal tissues in living animals) has made it highly amenable to many areas of research. *C. elegans* also has unique features, including a male/hermaphrodite sexual system, that make it particularly useful for studying muscle defects. Hermaphrodites have the ability to either self-fertilize or mate with a male. The self-fertilization of the hermaphrodite is internal and they do not need to move to feed or mate (Waterston, 1988). This is very useful when studying genes involved in muscle development as even completely paralyzed worms are still viable, fertile, and can be mated to a male capable of movement. This has led to the isolation of many mutations in *C. elegans* affecting movement (uncoordinated or *unc* mutants) (Brenner, 1973, 1974; Epstein and Thomson, 1974; Epstein et al., 1974; Waterston et al., 1974, 1977 and 1980) that would normally be lethal in other metazoans such as *Drosophila*. It was work done in *C. elegans* that provided the first complete sequence of a myosin heavy chain (Epstein et al., 1974; MacLeod et al., 1981; Karn et al., 1983; Dibb et al., 1985 and 1989) and the best evidence that thick and thin filaments show independent assembly (Epstein and Fischman, 1991). *C. elegans* has also allowed the discovery of new muscle proteins or proteins involved in the regulation of muscle development (Goetinck and Waterston, 1994; Benian et al., 1996; Venolia et al., 1999; Hresko et al., 1999; Ding and Candido, 2000). One of these genes, *unc-45*, (Epstein and Thomson, 1974; Venolia and Waterston, 1990) was shown to have a role in thick filament assembly, and mutant animals were paralyzed. Since the UNC-45 protein is conserved throughout metazoans (Hutagalung et al., 2002; Price et al., 2002), our lab has focused on determining what UNC-45 is doing in the assembly of thick filaments. Through the use of genetic and molecular manipulation of *C. elegans*, we will be able to determine its role in higher organisms.

The mechanisms governing muscle development and the regulation of muscle contraction are highly conserved across metazoans (Waterston, 1988; Moerman and Fire, 1997). The basic transcription factors governing myogenesis (Harfe et al., 1998b; Zhang et al., 1999; Corsi et al., 2000) as well as the process of myofibril assembly are similar in vertebrates and invertebrates (Barrall and Epstein, 1999; Crips et al., 1999; Gordon et al., 1999; Gregorio and Antin, 2000). Since most of the major proteins involved in the contractile apparatus are also conserved, it is expected that work done on these proteins in model organisms will be applicable to higher organisms. This will result in an increase in understanding of how muscle systems and diseases work in humans as well.

### 1.2. *C. elegans* body wall muscle structure

*C. elegans* has 95 body wall muscles used for locomotion, which are the only striated muscles found in the worm. Striated muscles act as a complex machine, containing specialized membranes made up of transverse tubules and the sacroplasmic reticulum, which in turn conveys a signal to the sarcomere (reviewed in Barral and Epstein, 1999). The sarcomere then generates force by converting the chemical energy of ATP to movement. The sarcomeres in the body wall muscles of *C. elegans* contain distributed attachments to the hypodermis along their length and are arranged longitudinally down the body in four parallel quadrants (Riddle et al., 1997) (Figure 1.1). The basic organization and structure of *C. elegans* muscles are similar to those of vertebrates, but there are some differences that will be discussed below (Waterston, 1988; Moerman and Fire, 1997). Striated body wall muscle cells can be divided into three distinct parts: (i) the cell body, which contains the nucleus and cytoplasmic organelles, (ii) the muscle arm, which extends from the cell body to the nerve cord for receiving synaptic input from the motor neurons, and (iii) the contractile myofilament lattice (described in detail below). A noticeable difference in organization compared to vertebrates is that the structural units (M lines and dense bodies) are offset from each other by more than a micron, rather than being in register with the filaments as in vertebrate muscles. The result of this is that the A (thick filament regions) to I (thin filament regions) striations are at an angle of 5-7° to the longitudinal axes of the filaments and the muscle cell, in comparison to 90° in vertebrate cross striated muscle. The oblique arrangement of the sarcomeres is thought to create a more evenly distributed muscle force and allows for smooth bending of the body instead of kinking (Burr and Gans, 1998). This allows for the sinusoidal movement necessary to propel the nematode.

A single muscle sarcomere in *C. elegans* is composed of alternating thick and thin filaments, M lines, and dense bodies (Z lines in vertebrate muscles) (Figure 1.2). The myosin containing thick filaments are stacked in the centre and aligned by the M lines, which are anchored to the membrane (Francis and Waterston, 1985). The thick filaments then interact with two sets of actin-containing thin filaments, which in turn are attached to the dense bodies at both ends (Waterston et al., 1980). The dense bodies are connected to the basement membrane by a transmembrane complex, which allows the transmission of contractile force from the hypodermis to the overlying cuticle (Moreman and Fire, 1997).

### *1.2.1. C. elegans thin filaments*

The main component of the thin filaments is actin, which forms a two-stranded helix that binds and activates myosin. Two actin binding proteins, tropomyosin and troponin lie along the groove between the two actin strands and regulate contraction in response to calcium (Moerman and Fire, 1997). Contraction results from the thin filaments sliding along the thick filaments.

### *1.2.2. C. elegans thick filaments*

Thick filaments are highly organized structures composed of a core of paramyosin surrounded by two isoforms of myosin (Figure 1.3). Myosin Heavy Chain A (MHC A) forms the inner region of the filament, while Myosin Heavy Chain B (MHC B) assembles on the remainder of the filament arms, extending the filaments to their proper length (see section 1.3.1 for more detail). These isoforms of myosin show distinct biochemical characteristics, including interactions with distinct sets of myosin binding proteins. Such differentiated assembly has not been achieved with purified myosin *in vitro* indicating the assembly of myosin into thick filaments *in vivo* requires the regulated interactions of as yet unknown factors. The myosin isoforms are arranged on a rigid inner tubular core that contains a layer of seven paramyosin subfilaments. These subfilaments are cross-linked by a set of inner rings composed of additional proteins, including the α, β and γ filagenins (Schmid and Epstein, 1998; Liu et al., 1998) (Figure 1.4). Paramyosin is primarily an α-helical coiled–coil rod composed of two identical subunits (Riddle et al., 1997) with non-helical ends that contain sites for interaction with other molecules. It is possible that these ends could be critical for thick filament assembly. In addition to acting as a structural template, the core is also thought to be involved in determining thick filament length, providing the proper length scaffold on which MHC B is assembled (Mackenzie and Epstein, 1980). Thick filaments show significant and regulated differences in their lengths and diameters, both changing during development as well as along an individual filament in an adult worm, with the diameter ranging from 33 nm in the center to 14 nm near the ends (Epstein et al., 1985). The three filagenins that co-assemble with paramyosin in the cores are expressed at distinct stages of muscle development (Liu et al., 2000). (Figure 1.4) α–filagenin is co-expressed with MHC A in the early embryonic myofibrils, whereas γ–filagenin is not detected until late embryogenesis, when myofibrils are well formed and of intermediate length. β-filagenin is detected only in larvae and adult worms and together with α–filagenin and γ–filagenin contributes to the assembly of long thick filaments. It is predicted that the filagenins help determine the length of paramyosin and that in turns determines how much MHC B spreads, thereby determining the length of the filament.

### 1.3. Muscle myosins

The two myosin isoforms, A and B, which make up the thick filament belong to the Type II class of myosins, otherwise known as conventional myosins. This class of myosin is involved in filament formation in both muscle and non-muscle cells. They are hexameric enzymes composed of two heavy chains and two pairs of light chains. Type II myosin molecules contain three functional domains: the head domain at the amino terminus, the neck, and a long coiled-coil rod at the carboxyl terminus, which homodimerizes to form a two-headed myosin (Figure 1.5). *C. elegans* type II myosins also contain a short non-helical tail at the carboxyl terminus that contains phosphorylation sites (Castellani et al., 1988). The tailpiece may be involved in filament organization in the late stages of embryogenesis (Hoppe et al., 2003). The head domain is the catalytic motor domain, containing actin-binding and ATPase activities that move thin filaments (actin) past thick filaments (myosin) to yield muscle contraction and movement of the animal. The neck domain contains helical sequences termed IQ motifs, which bind the myosin light chains and calmodulin (Cheney et al., 1992). The light chains are phosphorylated by myosin light chain kinase and change the conformation of the myosin head to facilitate actin binding. The rod domain of type II myosins dimerizes to form an $\alpha$-helical coiled coil that is the structural component responsible for the assembly of myosin into the thick filaments. By aiding in assembly, it also serves to anchor and position the motor domain so that it can interact with the thin filaments (actin) (Sellers, 2000).

### *1.3.1. Organization of myosins in the thick filament*

Thus, the thick filaments in *C. elegans* are composed of two different type II myosin isoforms: myosin heavy chain A (MHC A) and myosin heavy chain B (MHC B), which are differentially localized along the 10 $\mu$m long adult filament. The minor isoform MHC A, encoded by the *myo-3* gene, is located only in the central 2 μm of the filament and MHC B, encoded by the *unc-54* gene, is found along the length of the lateral arms (Miller et al., 1983, Figure 1.6). In addition to being differentially localized along the thick filament MHC A and MHC B have diverged to have distinct methods of packing to create a bi-polar filament. MHC A is capable of packing in anti-parallel fashion (tail to tail) (Miller et al., 1983), which creates a central bare region devoid of any myosin heads, where filament initiation is thought to occur. MHC B (which makes up the majority of the filament) is only able to pack in parallel fashion (head to tail) to create an extended myosin filament by adding molecules to either end of the central region. Genetic analysis has confirmed these functional differences, demonstrating that MHC A is uniquely capable of thick filament initiation. *myo-3* alleles (which eliminate MHC A function) cause embryonic paralysis and death due to the absence of a functional myofilament lattice, and the mutant animals contain no normal thick filaments (Waterston 1989). In contrast, *unc-54* (MHC B) homozygous null mutants are paralyzed, though viable and fertile, and exhibit disorganized body wall muscles containing a reduced amount of thick filaments (Epstein et al., 1986). Although disorganized, these filaments can be of normal length but contain MHC A along the entire length of the filament (Epstein et al., 1986). Even more striking, an increase in the amount of MHC A (provided by an over-expression of *myo-3* (*sup-3* alleles)) can rescue the movement and thick filament defects of *unc-54(0)* worms (Maruyama et al., 1989). This indicates that MHC A has the ability of initiating filament assembly as well as the ability to extend the filament in parallel when MHC B is absent. It is quite evident that MHC A and MHC B have different functions in filament assembly but it is not clear what parts of the sequence or tertiary structure could account for these differences.

Using molecular chimeras between MHC A and MHC B, Hoppe and Waterston (1996) were able to elucidate where MHC A filament initiation function lies within the molecule and how it is different from MHC B in that region. The chimeras contained complementary regions of MHC A and MHC B fused together to create a functional myosin molecule (as determined by rescuing an *unc-54(0)* mutation). The chimeras were then introduced into a *myo-3(0)* background to determine if filament initiation could occur. Two regions within the rod of MHC A were identified as potential sites required for filament initiation as determined by the ability to confer MHC A specific function (initiation) to the chimeric myosins (Figure 1.7). When sequence between MHC A and MHC B in these two regions was compared it was found that the hydrophobicity of the MHC A coat residues statistically increased only in those regions containing A-specific function (Hoppe and Waterston, 1996). In these regions, the MHC A values are similar to those of paramyosin, for which a more hydrophobic coat has been correlated with its more internal position within the thick filament (Cohen et al., 1987). It was also found that, in these regions, MHC A has other similarities to paramyosin that it does not share with MHC B, including fewer glycines residues and a higher P/K ratio (Hoppe and Waterston, 1996). It is believed that in paramyosin a reduced number of glycine residues result in a more rigid coiled-coil rod, which could be advantageous in filament initiation. It is now evident that MHC A has developed specialized structural roles that may be shared with paramyosin whereas MHC B has become specialized for other structural roles, such as elongation in parallel assembly. The increase in coat hydrophobicity on the surface of the rod, where protein-protein interactions occur, could help MHC A form more stable inter and/or intramolecular interactions with itself or paramyosin to allow initiation of the filament. Because of reduced coat hydrophobicity, antiparallel MHC B dimers may not be sufficiently stable to promote filament assembly.

Although it was originally thought that paramyosin would assemble first to form the core, recent genetic analysis has shown that MHC A forms first in the thick filament, without the need for paramyosin (Epstein et al., 1985, White et al., 2003). In contrast, MHC B synthesis and incorporation into nascent thick filaments is dependent on the presence of a properly assembled paramyosin-containing core (White et al., 2003). It is believed that the paramyosin core, along with MHC A, acts as a scaffold for the incorporation of MHC B into the elongating thick filament (White et al., 2003), although it is still unclear how MHC A initiates filament assembly. This, and many other questions regarding the molecular mechanisms of muscle filament assembly is the subject of ongoing research.

### *1.3.2. Assembly of myosin into thick filaments*

It is generally thought that the myosin rods are involved in assembly of the thick filament whereas the myosin head was the functional unit of the protein, mainly involved in generating movement. The ability to create movement is ATP-dependent and requires a direct interaction of the myosin head and actin. For this interaction to take place the myosin head must change its conformation to enable transformation of chemical energy from ATP hydrolysis into mechanical energy of myosin head movement along an actin filament. The change of conformation results in a contraction-relaxation cycle that is continuously repeated. Most of the mutations found in the myosin head in *C. elegans* have been in *unc-54* (MHC B) and not *myo-3* (MHC A), as the majority of the filament is composed of MHC B. One set of mutations at the *unc-54* locus supports this role for the head. These mutations affect the contraction-relaxation cycle in a manner that increases the duration of tight binding between myosin and actin (Moerman et al., 1982; Dibb et al., 1985). Most of these mutants have near normal muscle structure; however, they move slower than wild-type and seem to be stiffer, indicating a problem in muscle contractions. There are also dominant negative alleles located in the part of the *unc-54* gene encoding the head region of MHC B that indicate this region might be involved in filament assembly as well (Bejsovec and Anderson, 1988, 1990). Heterozygous *unc-54(d)* animals are paralyzed and have disorganized thick filaments (unlike the previously described mutations found in the head region), and homozygous animals are generally not viable and accumulate a very low level of MHC B (Bejsovec and Anderson, 1988). It is not clear how these alleles are having their effect. The *unc-54(d)* induced lethality cannot be due to a low amount of MHC B alone since homozygous *unc-54(0)* mutants are paralyzed but no lethality is observed. It has been proposed that the remaining MHC B in the *unc-54(d)* mutants has been altered and it acts as a poison for filament assembly (Bejsovec and Anderson, 1990), preventing any myosin from elongating the filament. One possible alteration that could occur to the MHC B head is incorrect folding, resulting in the inability of the myosin to assemble properly.

The core-folding motif is a 7 strand, mostly parallel, β sheet that is flanked by three α-helices on each side and forms the ATP-binding pocket. This structural motif is shared with the kinesin family of microtubule-based motors and with G-proteins involved in signal transduction (Rayment, 1996; Vale, 1996), all of which form very compact structures. Distinguishing it from kinesin motors, the myosin motif contains large insertions in the connecting loops of the core motif, which forms an extended actin-binding surface. This core motif is discontinuous and includes segments of primary structure from the entire length of the catalytic domain, which makes it much harder to fold than the structurally related kinesin motor domain. If the MHC B head is unable to fold correctly it could physically interfere with other myosins (e.g. paramyosin) forming the thick filament, resulting in a mutant phenotype worse than the *unc-54* null.

### *1.3.3. Myosin folding in the thick filaments*

Work done in cell culture on the amino terminal 845 residues of the mammalian myosin heavy chain (S1 fragment), which contains the motor domain and the light chain binding motifs, supports the mis-folded myosin head hypothesis observed in *unc-54(d)* worms. Light chain binding, folding, and dimerization of the rod domain (S2 fragment) preceded motor domain folding so it was inferred that motor domain folding was the rate-limiting step in the assembly of myo-filaments. When the S1 fragment was synthesized in a rabbit reticulocyte lysate, very slow folding kinetics and thermal instability were observed for the motor domain. Folding kinetics were examined in a variety of cell types and it was only when the S1 fragment was expressed in skeletal muscle cells that a properly folded and functional myosin was observed (Chow et al., 2002). This indicates that there are muscle specific factors in the folding pathway of the motor domain. When the myosin folding pathway was characterized in more detail in myocytes it was found that the immature myosins go through a variety of intermediates ranging from bright globular foci, short filamentous structures, and finally to myofibrils (Srikakulam and Winkelmann, 2003). Immunostaining of these intermediates revealed that they co-localize with two molecular chaperones, Hsp90 and Hcs70, while the myosin already assembled into striated myofibrils showed no presence of these chaperones (Srikakulam and Winkelmann, 2003). Since molecular chaperones selectively bind unfolded proteins (reviewed in Agashe and Hartl, 2000; Young et al, 2001), the co-localization of Hsp90 and Hsc70 with the myosin in the intermediates but not the mature myofibrils suggests they are folding intermediates. Conformation sensitive antibodies were used to determine that these intermediates have a fully folded myosin rod but only a partially folded motor domain (Srikakulam and Winkelmann, 2003). This evidence suggests it is the myosin motor domain that requires the action of the molecular chaperones to complete folding and allow incorporation into the mature myofibrils (Srikakulam and Winkelmann, 2003). This is supported by the evidence that both Hsp90 and Hsc70 co-immunoprecipitated with denatured myosin S1 fragment and both chaperones were absent in reactions lacking either the anti-S1 antibody or the myosin S1 itself (Srikakulam and Winkelmann, 2003). These chaperones could prevent release of active, unregulated misfolded motors in the cytoplasm that would otherwise interfere with normal myofilament assembly, potentially what is happening in the *unc-54(d)* mutations.

Hsc70 and Hsp90 are major molecular chaperones, present in the cytosol and the nucleus, both in muscle and non-muscle cells, involved in processes ranging from protection of proteins from cellular stress (Hsc70) (Naylor and Hartl, 2001) to folding and activation of signal transduction kinases (Hsp90) (Buchner, 1999; Caplan, 1999). These chaperones could require muscle specific adaptors for efficient targeting and folding of myosin. Such an adaptor would have to be localized to muscle cells and possibly act as a co-chaperone to help Hsp90 and Hsc70 correctly fold myosin. This factor might also associate with the mature myosin and help direct formation of the filament once the myosin is properly folded, as Hsp90 and Hsc70 are not needed for this step. It is known that many Hsp90 and Hsc70 co-chaperones share an N-terminal domain containing a set of repeats termed the tetratricopeptide repeats (TPR motif) that bind a C-terminal recognition peptide of Hsp90 and Hsp70/Hsc70 (Scheufler et al., 2000). Preliminary characterization of the UNC-45 protein, which was initially identified in *C. elegans,* revealed it was expressed in muscle cells and contained a set of TPR repeats at its amino terminus. Evidence suggests that it could be acting as a muscle adaptor used to recruit chaperones and/or a co-chaperone itself to help the myosin molecules correctly fold and assemble into the thick filaments of myofibrils (Barral et al., 1998; Ao and Pilgrim, 2000; Barral et al., 2002). Testing some of the hypotheses provided by this theory, and developing the molecular reagents required for further investigations, are the objectives of the present work.

### 1.4. *unc-45* genetic analysis

A temperature-sensitive and recessive allele of *unc-45, e286,* was identified through the use of an ethyl methanesulphonate mutagenesis screen looking for uncoordinated animals (unc) (Brenner, 1973, 1974; Epstein and Thomson, 1974). In contrast to wild-type nematodes, which are highly motile at both 25°C and 16°C, homozygous *unc-45(e286)* animals are paralyzed at 25°C with very slow body movements and a smaller brood size. However, if grown at 16°C, the movement and brood size of *unc-45(e286)* mutants are indistinguishable from wild-type (Epstein and

Thomson, 1974). It was also reported that if the temperature was shifted either way during larval development (before L4 stage) a complete reversal of phenotype from mutant to wild-type and vice versa was observed (Epstein and Thomson, 1974). It is during this time in development that the majority of myofilament assembly occurs. Upon closer examination, it was found that these mutant worms, when grown at 25°C, have very disorganized sarcomeres with no distinguishable A-bands, I-bands, or dense bodies, and a reduced number of recognizable thick filaments. This observed phenotype and the observation that it can reversed during larval development indicates that UNC-45 could be playing a role in the assembly of thick filaments in the body wall muscles during muscle development.

The subsequent isolation of recessive lethal alleles of *unc-45* demonstrated that it is an essential gene necessary for normal development (Venolia and Waterston, 1990). Embryos homozygous for the lethal alleles lack all muscle contractility and fail to elongate, arresting with a "pat" phenotype (paralyzed and arrested at two-fold stage). This phenotype is seen in many mutants of genes involved in body wall muscle function and assembly (e.g. *myo-3(0)*) (Williams and Waterston, 1994). The embryos also never begin pharyngeal pumping indicating a defect in pharyngeal muscle function. One of the lethal alleles (*unc-45(st604)*) has presumed partial activity as the embryos show some contractile function. *st604* homozygotes are also subject to full maternal rescue, indicating the *unc-45* gene product (mRNA or protein) is present in the oocytes (Venolia and Waterston, 1990).

In the body wall muscles, the paralysis of *unc-45* mutants is presumably due to the severely disorganized thick filaments arrays, indicating a potential interaction with the myosins that compose the thick filament. Embryos homozygous for some *unc-45* lethal alleles are able to begin MHC A assembly (initiation of filament assembly) but cannot complete the remainder of the filaments (which are composed of MHC B). This suggests that UNC-45 could be involved in regulating assembly of MHC B into the thick filament by potentially acting as a [co-]chaperone (as mentioned above). If this is the case, the MHC B present in the *unc-45* mutants would be abnormal or non-functional and unable to assemble into the thick filament properly, resulting in paralyzed animals.

## 1.5. Molecular analysis of UNC-45

### *1.5.1. Localization of UNC-45 in the body wall muscles*

Analysis of UNC-45 localization (directly or by using reporters) has supported the genetic evidence suggesting it is involved in thick filament assembly. An *unc-45* promoter driving expression of GFP resulted in muscle-specific expression in the pharynx, body wall, vulval, and anal depressor muscles (Venolia et al., 1999) (Figure 1.8A). To look more closely at the body wall muscles, a stable transgenic line of *unc-45* cDNA::GFP was created which can rescue the *unc-45(ts)* mutant phenotype at the restrictive temperature of 25°C (Ao and Pilgrim, 2000). UNC-45::GFP expression pattern in the body wall muscles resembles the pattern of the A-bands of the thick filaments (Figure 1.8B) suggesting UNC-45 interacts with components of the thick filament (i.e. myosin) (Ao and Pilgrim, 2000). Antiserum generated against UNC-45 (58 residue region from amino acid 18 to 76 of the predicted protein) supported the localization to the thick filament but it also added another level of detail. UNC-45 was shown to co-localize with only the MHC B region of the thick filament (outer region) leaving an unstained central gap where MHC A is present (Ao and Pilgrim, 2000, Figure 1.9). Mutant UNC-45 protein was still associated with the disorganized thick filaments in *unc-45(ts)* worms but differential localization could not be confirmed because MHC A and MHC B are no longer located in separate regions of the thick filament in these worms, as antibodies against either show the same pattern (Ao and Pilgrim, 2000). UNC-45 was not localized to the thick filaments in *unc-54(0)* and *unc-54(0);sup-3* worms in which the thick filaments are made entirely of MHC A (Ao and Pilgrim, 2000). These results suggest that MHC B needs UNC-45 activity in order to assemble into the thick filament and that MHC A assembly into the thick filament is an UNC-45 independent process. In the absence of UNC-45, MHC B cannot properly form and MHC A must make up the remainder of the filament, which allows the worms to survive to adult, although they are paralyzed. When UNC-45 localization was examined in the larval stages an interesting observation was noted for the L1 stage, when the body wall muscles are just starting to form (Figure 1.10). At this point the MHC B as has already been assembled into thick filaments near the cell membrane, whereas UNC-45 is still mainly diffuse in the cytoplasm (Ao and Pilgrim, 2000). This could indicate that UNC-45 is not needed or does not need to localize directly to the thick filament in the early stages of filament formation and that its co-chaperone activities are needed later in the assembly pathway of MHC B.

From the observation that both *myo-3(0)* and *unc-45* lethal mutations share the same pat phenotype it also seems possible that UNC-45 could be required for some aspect of MHC A assembly and initiation in the very early stages of muscle development.

Although there has been no direct evidence that UNC-45 and MHC A interact in the adult filament there could be an involvement in the embryo that has not yet been characterized.

### *1.5.2. Localization of UNC-45 in the early embryo*

As mentioned above, lethal alleles of *unc-45* result in embryonic lethality (Venolia and Waterston, 1990) with the strongest lethal alleles lacking any muscle contractions and arresting with a pat phenotype. While it is possible that this phenotype is due to as yet uncharacterized interactions with MHC A during initiation of body wall assembly (described above in section 1.5.1), there is another possibility for the embryonic lethality seen. Whereas muscle cell components are produced zygotically, embryonic-lethal alleles of *unc-45* show terminal phenotypes that are dependent on the genotype of the mother indicating that the *unc-45* product is contributed through the oocyte maternally (Venolia and Waterston, 1990). Immunostaining with UNC-45 in early embryos (two-cell stage) revealed that UNC-45 was expressed in all cells concentrated at the cell cortex (Kachur et al., 2004). Zygotic expression of UNC-45 is only seen in muscle specific cells (Venolia et al., 1999), indicating the expression in the early embryo is from a maternal contribution.

When animals were produced with a reduced amount of maternal UNC-45 they showed defects much earlier than the previously reported pat phenotype. The most common defect was a failure to complete cytokinesis during the first cleavage divisions in the early embryo, resulting in polyploid daughter cells (Kachur et al., 2004). The localization pattern for UNC-45 at the cell cortex is consistent with a role in cytokinesis, which forms a cleavage furrow at cell cortexes. Since UNC-45 has been shown to co-localize with a type II myosin in the body wall muscles, potentially to help it fold, it is reasonable to think that UNC-45 could have a similar role in helping myosins involved in cytokinesis in the early embryo. NMY-2 is a type II non-muscle myosin that is localized to the embryonic cleavage furrow of the two-cell embryo and is required for polarized cytoplasmic flow and cytokinesis (Guo and Kemphes, 1996; Shelton et al., 1999). Yeast two-hybrid analysis was performed with UNC-45 to determine putative interacting proteins and three strong positives were identified in the screen. One of them was NMY-2, another was an uncharacterized Type V myosin, HUM-2, and the last was the *C. elegans* homolog of UFD-2 (described in Section 1.8) (Kachur et al., 2004). NMY-2 and UNC-45 were shown to co-localize in the same embryo and UNC-45 localization was disrupted when NMY-2 was absent (Kachur et al., 2004). This relationship between UNC-45 and NMY-2 is strikingly similar to that of UNC-45 and MHC B in the body wall muscles, where UNC-45 localization is dependent on the presence of MHC B (Ao and Pilgrim, 2000). Due to these similarities it is conceivable that UNC-45 also aids in assembly or localization of NMY-2, a non-muscle type II myosin, to allow for proper cytokinesis during the early stages of embryo development. *unc-45* mutants depleted of maternal UNC-45 could be unable to complete cytokinesis due to the mis-assembly or mis-localization of NMY-2. It is also interesting to hypothesize that UNC-45 could have a role in more than just the assembly of type II myosins, as the type V myosin, HUM-2, was pulled out in the yeast two-hybrid screen and recently, a homolog of UNC-45 in yeast was shown to interact with a type V and a type I myosin as well (see section 1.6.2). The role of UNC-45 would therefore become much more complicated as these classes of myosins have a very divergent structure from the Type II myosins (Titus, 1997). UNC-45 would have to adopt a unique method of folding and binding for each class of myosin.

## 1.6. Protein structure of UNC-45

To complete the understanding of the genetic and molecular observations of UNC-45 the specific role of the protein must be examined in more detail. The predicted UNC-45 protein consists of 961 amino acids and has three main domains. At the amino terminus there are three tandem tetratricopeptide (TPR) motifs, then a central domain of unknown function, but which is highly conserved among UNC-45 homologs in metazoans. The carboxyl region contains a domain shared with metazoan and fungal homologs (UCS domain), and is discussed in detail below (see section 1.6.2) (Figure 1.11). This domain is believed to be the functional part of the protein and through database searches it was found that a variety of other proteins share this domain.

### *1.6.1. TPR domain*

TPR repeats are modules of 34 amino acids, found in tandem arrays of 3 to 16 repeats, in a diverse set of proteins, and which are known to be involved in protein-protein interactions (Blatch and Lassle, 1999). Most TPR-containing proteins are associated with multi-protein complexes involved in chaperoning, cell-cycle, transcription, and protein transport complexes. The TPR domain of UNC-45 shares sequence similarity with that of Hop (Hsp70/Hsp90-organizing protein), which binds conserved sites in the molecular chaperones Hsp70 and Hsp90 (Barral et al., 1998;

Venolia et al., 1999). All three of these proteins have TPR repeats through which they are believed to interact. Barral et al. (2002) also showed that full-length UNC-45 could be co-immunoprecipitated by endogenous Hsp90 and that the interaction disappeared when the TPR domain of UNC-45 was removed. Hsp70 also was shown to have an interaction with the TPR domain but it was much weaker than Hsp90, indicating it could potentially be involved in the complex as well. These results suggest that UNC-45 is acting as a co-chaperone to bring Hsp90 (by binding to its TPR repeats) into contact with the myosin head to be folded by a multi-chaperone complex.

Hsp90 is unique among molecular chaperones in that it seems to have much more specific targets and a unique protein folding pathway than the more promiscuous chaperones (for example Hsp70 and the chaperonins). The majority of Hsp90's substrates are signal transduction proteins, for example steroid hormone receptors and signaling kinases (Picard et al., 1990; Xu and Lindquist, 1993). A new substrate member, myosin, has also recently been identified that expands that role of Hsp90 chaperone activity (as described above) (Barral et al., 2002). Most of the substrate proteins to which Hsp90 binds are in a near native state and thus at a later stage in folding (Jakob et al., 1995), indicating Hsp90 could be acting at the last step in the specialized folding pathway after the more general chaperones have performed their function. Hsp90 is an ATP-dependent chaperone (Prodromou et al., 1997; Obermann et al., 1998; Panaretou et al., 1998) but the exact mechanism of the ATPase activity is still under investigation. The current theory is that in the ATP-bound state, the Hsp90 dimer forms a "molecular clamp" around the substrate to stably bind it. Hydrolysis of the bound ATP releases the substrate by opening up the Hsp90 dimer or some other conformational change (Chadli et al., 2000; Young and Hartl, 2000; Young et al., 2001).

As mentioned above, the TPR domain of UNC-45 is similar to that of Hop so it is possible that UNC-45 is performing a similar function to Hop but has become specialized to interact with myosins and help them fold. One of the specific systems in which Hop is involved is the assembly and maintenance of steroid receptor complexes. Both progesterone (Smith, 1993) and glucocorticoid (Scherrer et al., 1990) receptors depend on proper assembly with Hsp90 and other chaperone proteins to maintain high affinity hormone binding at physiological temperatures. Hop has been shown to participate at an intermediate assembly stage that is essential for the maturation of functional complexes. To determine the role of Hop, point mutations were made in the conserved TPR domains that had previously been shown to bind Hsp90 and Hsp70 (Chen et al., 1996; Lassle et al., 1997) so that a Hop deficient in Hsp90 and Hsp70 binding was produced (Chen et al, 1998). It was found that Hop acts as an adaptor that directs Hsp90 to preexisting Hsp70-steroid receptor complexes and without it Hsp90 could not bind to the complex and maturation could not occur (Chen et al., 1998). UNC-45 could be acting as a similar adaptor in muscle cells to bring MHC B into contact with Hsp90 and possibly Hsp70 (both of which co-purify with UNC-45) as it progresses through its folding stages (Figure 1.12). Although evidence points to UNC-45 functioning the same way as Hop, UNC-45 has characteristics that are not found in Hop which could have evolved specifically for the folding of myosin heads. Hop is never seen in the mature complexes whereas UNC-45 remains localized to MHC B in the mature thick filaments of *C. elegans*. This could indicate that MHC B requires the presence of a chaperone even once it has been assembled and UNC-45 remains in contact with it to recruit the chaperones as soon as they are needed. The myosin head is undergoing constant conformational changes that require a lot of energy and therefore have a high probability of becoming mis-folded in the mature form. The heads also need to function very efficiently in order to create movement so mis-folded myosins must be corrected very quickly. UNC-45 has also evolved intrinsic chaperone activities (see Section 1.6.2) that could be due to the increased need for chaperone activity in folding of the myosin head.

Recently, another folding pathway similar to the UNC-45/Hsp90/MHC B pathway has been identified involving a cyclin-dependent kinase inhibitor, p21 (Jascur et al, 2005). p21 is a short-lived protein with a loosely folded, ill-defined structure that is efficiently degraded by the proteasome (Kriwacki et al., 1996; Blagosklonny et al., 1996). WISp39 is a newly identified protein with a TPR domain resembling that of Hop that has been shown to stabilize newly synthesized p21 by preventing proteasomal degradation. The stabilization is achieved by the recruitment of Hsp90 through the TPR domain of WISp39 to bring the chaperone into contact with p21 (Jascur et al., 2005). Although this is a completely unrelated system from myosin folding, a very similar approach to help with the folding and stabilization of problem proteins has evolved. WISp39 seems to be working in a manner similar to UNC-45 to act as a co-chaperone to bring Hsp90 into contact with its client proteins (p21 and MHC B respectively). The evidence that other proteins also function to bring Hsp90 and Hsp70 to their target substrates supports the hypothesized role of UNC-45 acting as a muscle specific adaptor/co-chaperone for MHC B.

### *1.6.2. UCS domain and fungal homologs*

The UCS domain of UNC-45 is believed to be the most crucial part of the protein. The C-terminal domain of UNC-45 is conserved in a variety of fungal proteins (Figure 1.13). This family of conserved proteins was named for the first three members identified: UNC-45 in *C. elegans*, CRO1 in *P. anserina (*Berteaux-Lecellier et al., 1998), and She4p in *S. cerevisiae* (Jansen et al., 1996). Recently, another member of the family, Rng3p has been identified in *S. pombe* (Wong et al., 2000). By looking at the functions of the fungal homologs we can propose a molecular role for the UCS domain in assembly of MHC B that supports a role for chaperone activity. Although their mutant phenotypes appear to have few significant similarities, all of these proteins are linked together by their common association with processes related to, or requiring, myosins (Table 1.1)

Three of the four temperature sensitive alleles are associated with missense substitutions in the UCS domain and two lethal alleles contain stop codons in the central domain, which creates a genetic null lacking the UCS domain (Barral et al., 1998). Since the temperature sensitive alleles produce unstable scrambled filaments, the UCS domain is directly implicated in thick filament assembly and interactions with myosin. Barral et al., (2002) found that the Central/UCS domain interacted with the S1 scallop myosin head fragment *in vitro,* supporting the importance of this domain. They also found that this interaction required above ambient temperatures *in vitro*, which is consistent with a chaperone:substrate relationship. Upon closer examination of the UCS domain they also found that it exhibited characteristics common to chaperones, for example, preventing aggregation of partially unfolded proteins and maintaining partially unfolded proteins in a state competent for refolding (Barral et al., 2002). These experiments provided the first direct evidence that UNC-45 could be acting as a (co)-chaperone for MHC B.

### She4p in *S. cerevisiae*

*S. cerevisiae* She4p is involved in two myosin-dependent events: endocytosis and mRNA localization (Wendland et al., 1996; Long et al., 1997) and *she4* mutants have impaired receptor-mediated endocytosis, temperature sensitive growth, poorly organized cortical cytoskeletons, and a defect in actin polarization (reviewed in Wesche et al., 2003). In contrast to other UCS proteins, which interact with Class II myosins, the observed phenotypes of *she4* mutants are similar to mutations reported in yeast Class I (Myo3p and Myo5p) and Class V (Myo4p) myosins. The majority of these phenotypes are caused by the loss of myosin-actin association as a result of myosin mis-distribution, implicating She4p in myosin distribution within the cell. Wesche et al. (2003) supported this idea by showing that She4p binds to the motor domains of class V myosin Myo4p and class I myosin Myo5p, an interaction that depends on She4p's UCS domain. Mutation analysis of She4p also showed it is required for proper myosin localization and function, including the ability to bind to filamentous actin (Wesche et al., 2003) and might be acting as a regulator of myosin function possibly by supporting efficient actin binding. She4p binds to Myo5p in a temperature sensitive manner (Toi et al., 2003). The amount of She4p that bound Myo5p was dramatically increased at 30°C versus 4°C (Toi et al., 2003), which is consistent with a chaperone interacting with its substrate and similar to what was found for UNC-45 (Barral et al., 2002). These observations indicate UCS proteins are not class II myosin-specific factors as originally thought but are more general factors involved in a wide variety of myosin assembly, localization, and possibly chaperone processes that are very divergent across phyla.

### CRO1 in *Podospora anserina*

The CRO1 gene of *P. anserina* was identified through a screen for defects in sexual sporulation and the protein shares 21% identity and 40% similarity with She4p. CRO1 regulates the switch from vegetative growth, where nuclei are in syncytia, to the cellular stage required for sexual reproduction (Berteaux-Lecellier et al., 1998). The *cro1-1* null allele shows a variety of phenotypes including abortive meioses leading to polyploid nuclei, an inability to form septa between the daughter nuclei following mitotic division, and decreased filamentous growth. One phenotype that could be significant to the common thread of UCS proteins is that the syncytial cytoplasm becomes filled with multiple nuclei and the actin cytoskeleton becomes disorganized (Berteaux-Lecellier et al., 1998), as a similar phenotype is seen in *she4* mutants. Spindle positioning is random and both actin belts and septa are misplaced or absent. It is possible that in the absence of CRO1 function, myosins that interact with and organize the actin cytoskeleton may not be functional and as a result, the signaling pathway that regulates actin assembly and microtubule disassembly is disrupted (Epstein et al., 2002). CRO1 could be helping to properly assemble and regulate the myosin to bring it into contact with the actin cytoskeleton to proceed through to the cellular state.

### Rng3 in *Schizosaccharomyces pombe*

Rng3p is thought to be the most similar fungal homologue to *C. elegans* UNC-45 due to its interaction with a conventional type II myosin, Myo2p, which is the same type of myosin as MHC B. Rng3 is required for the proper assembly and localization of

Myo2p and F-actin into a functional actomyosin ring (Wong et al., 2000). The actomyosin ring is assembled at the medial cortex upon entry into mitosis and constricts at the end of anaphase to guide the centripetal deposition of the septum to allow for cytokinesis and completion of cell division in *S. pombe*. Lord and Pollard (2004) showed that purified Myo2p retained full ATPase activity and the ability to bind to actin filaments, however it could not support the gliding motility of the actin filaments. With the addition of the UCS domain of Rng3 alone, the motility of the actin filaments was restored. This indicates Rng3 is essential for function of the active Myo2p motor by promoting an efficient interaction of Myo2p with the actin filaments, which is essential for the formation of a competent actomyosin ring. There is also a specific mutant of Myo2p, *myo2-E1*, which causes a substitution in the motor domain that sequesters wild-type Rng3 to the defective actomyosin ring. Rng3p could be sequestered to try and help maintain this mutant in an assembly competent state, acting similar to UNC-45 in the body wall muscles of *C. elegans*.

It is interesting to note that Rng3p concentrates in the contractile ring after Myo2p, which is similar to the observations in *C. elegans* thick filaments in which UNC-45 co-localizes to the sarcomere after incorporation of MHC B. The chaperones Hsc70 and Hsp90 only co-localize with myosin II before their incorporation into sarcomeres (Srikakulam and Winkelmann, 2004). Purified Myo2p (without Rng3) also binds reversibly to actin filaments and has robust ATPase activity, so it must be folded without associated Rng3p (Lord and Pollard, 2004). This evidence supports the idea that UCS proteins could be acting as more than chaperones, possibly playing a role in myosin activation, which would occur after the myosin has been fully folded. Another explanation is that UCS proteins remain localized with their myosin counterparts to always be ready to recruit chaperones to a myosin that has become mis-folded, which would reduce the lag time required for fixing an essential protein.

By analyzing the function of the fungal UCS homologs we are learning that UCS proteins do not act only on conventional Type II myosins in muscle assembly, but also on Type I and V myosins involved in cytokinesis and cellular trafficking. Some evidence points to them acting as chaperones or co-chaperones to help them fold, while other evidence seems to support a role in myosin activation. Whatever the exact role turns out to be it is evident that they have evolved to assist myosins, which need help in either assembling or maintenance of a functional state.

### 1.7. Vertebrate homologs of UNC-45

So far I have discussed evidence that *C. elegans* UNC-45 and the UCS fungal homologs all have a role in myosin organization, possibly by functioning as co-chaperones to recruit Hsp90 and Hsp70 to help assemble and activate the myosin head. Chaperones in general are widely expressed throughout various tissues and many eukaryotes share a common set of chaperones (reviewed in Hartl and Hayer-Hartl, 2002). Thus, it was predicted that other metazoans besides *C. elegans* are likely to carry homologues of UNC-45. Sequence homologues can be found in many genome databases (Hutagalung et al., 2002 and Ao, 2001). The metazoan UCS proteins are also termed UNC-45, since the first identified metazoan UCS protein was *C. elegans* UNC-45. The metazoan UNC-45 proteins are much more similar to *C. elegans* UNC-45 than the fungal UCS proteins because they contain N-terminal TPR repeats similar to *C. elegans* UNC-

45, which are absent in the fungal UCS family members. The three vertebrate homologs studied in the most detail are found in *Danio rerio, Mus musculus,* and *Homo sapiens.* While only a single UNC-45 homolog has been found in invertebrates two copies of UNC-45 seem to have evolved in vertebrates and appear to have divergent functions. One UNC-45 homolog appears to be expressed ubiquitously (general cell or GC form), while the other one is a muscle specific homolog (striated muscle or SM form) (Etheridge et al., 2002; Price et al., 2002).

### *1.7.1. Mammalian homologs of UNC-45*

In the mouse, the two isoforms of UNC-45 are differentially expressed. GC UNC-45 mRNA was detected in wide range of tissues during all stages of development, indicating a multi-organ expression pattern (Price et al., 2002). The SM UNC-45 mRNA was very abundant in skeletal muscle and the heart, both of which consist predominantly of striated muscle fibers (Price et al., 2002).

In human cell lines undergoing muscle differentiation only the GC UNC-45 mRNA was expressed in the proliferating non-differentiating myoblasts, while the SM UNC-45 mRNA was first expressed when the myogenic cells were actively fusing and differentiating (Price et al., 2002). At this time the young myotubes are in the process of assembling and remodeling their myofibrils (thick filament assembly and sarcomere formation). Reduction of the mRNA of SM UNC-45 directly affected sarcomere formation so that half the myotubes lacked striated myofibrils, indicating a role in striated muscle differentiation. A reduction in GC UNC-45 mRNA suppressed cell proliferation and inhibited myoblast fusion, suggesting an involvement in cytoskeleton maintenance of myofibrils (Price et al. 2002). These *in vitro* experiments are consistent with the SM UNC-45 homologue having specificity to the muscle MHC II similar to that seen in *C. elegans*. This work also suggests the GC UNC-45 homologue has specialized towards the interaction with non-muscle myosins. These two different roles of vertebrate UNC-45 homologs seem to be carried out by a single protein molecule in *C. elegans* (See section 1.5.1 and 1.5.2).

### *1.7.2. Danio rerio UNC-45*

As in mammals, *Danio rerio* (zebrafish) also has two isoforms of UNC-45. The *Dr* GC *unc-45* is ubiquitously expressed with potential vascular enrichment. The *Dr* SM *unc-45* is expressed in all striated muscle, including the slow and fast twitch muscle, pectoral fin and jaw muscle, the heart primordium and the developing heart (Etheridge et al., 2002; Serene Wohlgemuth, personal communication). In very early heart development SM *unc-45* is expressed in both the atrium and ventricle but following looping of the heart it is only expressed in the ventricle (Serene Wohlgemuth, personal communication). A morpholino oligonucleotide (MO) complimentary to SM *unc-45* was constructed to determine the mutant phenotype of the gene. A morpholino is a chemically modified oligonucleotide that physically blocks translation of RNA by binding to the translation start site (Nasevicius and Ekker, 2000) and can phenocopy a null mutation. Embryos treated with the MO were paralyzed with no blood circulation. There is a lack of ventricular function in the heart, which could be caused by the lack of circulation, but the atrium appears normal. The heart also does not loop, which is also likely due to the lack of circulation (Serene Wohlgemuth, personal communication). When the striated muscle was examined in the morphants (MO treated embryos) it appears to be completely disorganized with a lack of muscle striation. This phenotype is very similar to what is observed in *C. elegans unc-45* mutants indicating that the *Dr* SM UNC-45 could be playing a similar role to help assemble and fold the myosins in zebrafish muscle. There are also heart specific myosins present in zebrafish that could require UNC-45 for folding. The lack of UNC-45 in the morphants could result in improper organization and folding of the heart myosins leading to the defects observed in the heart and circulatory system. This data suggests that UNC-45 has a conserved role in the organization of myosins but has also expanded the role through the GC form of UNC-45, potentially for involvement in vascular and cytoskeletal maintenance.

### 1.8. Regulation of UNC-45

If UNC-45 does act as a co-chaperone for myosins it would require very tight regulation to insure the correct amount of myosin gets folded and assembled into the thick filament. Recently, Hoppe et al., (2004) have found a novel pathway for the regulation of UNC-45. Following up on some observations from our lab (Ao, 2001) UNC-45 was shown to interact with UFD-2 and CHN-1, which are both components of the ubiquitin degradation system. UFD-2 was first identified in yeast as a new member of the ubiquitin pathway (Koegl et al., 1999). It acts as an additional conjugation factor (termed E4) and catalyzes multiubiquitin chain assembly on target substrates (Koegl et al., 1999) (Figure 1.14). CHN-1 is the *C. elegans* homolog of CHIP, a co-chaperone that contains three tandem TPR motifs at its N-terminus that bind to the chaperones Hsp70 and Hsp90. CHIP also displays E4 ubiquitin enzyme function and substrates ubiquitinated by CHIP undergo accelerated degradation by the 26S proteasome (Connell et al., 2001). It was postulated that CHIP could assist in regulating the cellular balance between folding and degradation (Cyr et al., 2002; Murata et al., 2003). The complex formed by CHN-1 and UFD-2 multiubiquitinates UNC-45 and thus functions as a novel E3/E4 complex that regulates UNC-45 protein levels (Hoppe et al., 2004) (Figure 1.15). The CHN-1/UFD-2 complex appears to be a negative regulator of UNC-45 by targeting it for degradation. The movement defects of *unc-45(ts)* mutants can be suppressed in animals lacking CHN-1, due to an increases in the stability of the UNC-45(ts) protein, whereas over-expression of CHN-1 causes an increase in severity of the *unc-45(ts)* phenotype (Hoppe et al., 2004). The organization of myosin into cellular structures, specifically the thick filaments in *C. elegans*, requires precise spatial and temporal control, as well as the help of muscle specific chaperones. Due to the special requirements of myosin, UNC-45 (its muscle specific chaperone) must require stringent regulation to ensure myosin is incorporated properly and at the right time. It now appears this regulation is dependent on the ubiquitin degradation system, mediated through CHN-1 and UFD-2 (which could ensure only properly folded and fully functional UNC-45 is present when myosin needs to be folded).

### 1.9. Goals of this thesis

When I started my Masters project, it was known that UNC-45 co-localizes with MHC B in the thick filaments of the body wall muscles of *C. elegans*. In *unc-45* mutants, thick filament organization was disrupted, resulting in paralyzed animals. During the course of my work it was also found that UNC-45 has intrinsic chaperone activities and acts as a co-chaperone to recruit Hsp90 and Hsp70 to myosin. An *in vitro* experiment showed that UNC-45 bound to the S1 fragment of myosin (created from a cleavage reaction of scallop muscle resulting in the head domain and light chain binding domain of the myosin molecule) (Barral et al., 2002). My goal was to determine how and if this interaction occurred *in vivo*. I obtained a series of strains containing complementary chimeric fusions of MHC A and MHC B myosins (kindly provided by Dr. Pamela Hoppe). By using the UNC-45 antibody already created (Ao and Pilgrim, 2001), I could stain the thick filaments of these transgenic worms. If UNC-45 is still localized to the thick filament it will mean the chimeric myosin contains the region of MHC B to which UNC- 45 binds, as UNC-45 is only associated with MHC B and not MHC A *in vivo* (assuming that the binding of UNC-45 to the MHC B thick filament *in vivo* is due to a relatively small region of the MHC B molecule). Conversely, if UNC-45 is not localized to the thick filaments, it will indicate that the binding site of UNC-45 is no longer present. I hypothesized that the binding site for UNC-45 would be in the head region of MHC B as it has been well documented that the heads of myosin molecules need the help of muscle specific chaperones to fold properly. This would also support the *in vitro* data found for the S1 fragment (Barral et al., 2002).

I wanted to determine if there was a specific region of MHC B that required UNC-45 expression or if the entire molecule needed it for assembly. When doing this, I would also determine whether UNC-45 expression was needed for the assembly of MHC A, as discussed earlier. To do this, I intended to cross the transgenic strains (*unc-54(0)*+chimera) with *unc-45(ts)* worms to determine if the double mutant was able to move at the restrictive temperature. I predicted that worms containing the MHC B head in the absence of UNC-45 would be paralyzed while worms containing the MHC A head would be able to move normally. We assumed that the MHC A head did not require UNC-45 chaperone activity to fold properly while the MHC B head was dependent on it.

### 1.10. Summary of this thesis

As mentioned above, the majority of my experiments depended on the UNC-45 antibody that was created in our lab (Ao and Pilgrim, 2000). Unfortunately, when I went to use this antibody, I was not able to obtain specific and sensitive staining. I attempted to re-purify frozen serum; however, I could not reconstitute the antibody. I therefore spent the majority of my time trying various methods to create a new UNC-45 antibody with a strong and reproducible signal. Once I got the antibody and confirmed that it reacted as expected, I was able to stain the transgenic worms carrying the chimeric myosins and look for UNC-45 localization. I determined that UNC-45 requires the rod region of MHC B to localize to the thick filament, and not the head region as predicted. I also determined that transgenic worms that contained the MHC A head instead of MHC B were able to move in the absence of *unc-45*, indicating that MHC A head assembly, at least scored by movement of the animal, is independent of UNC-45

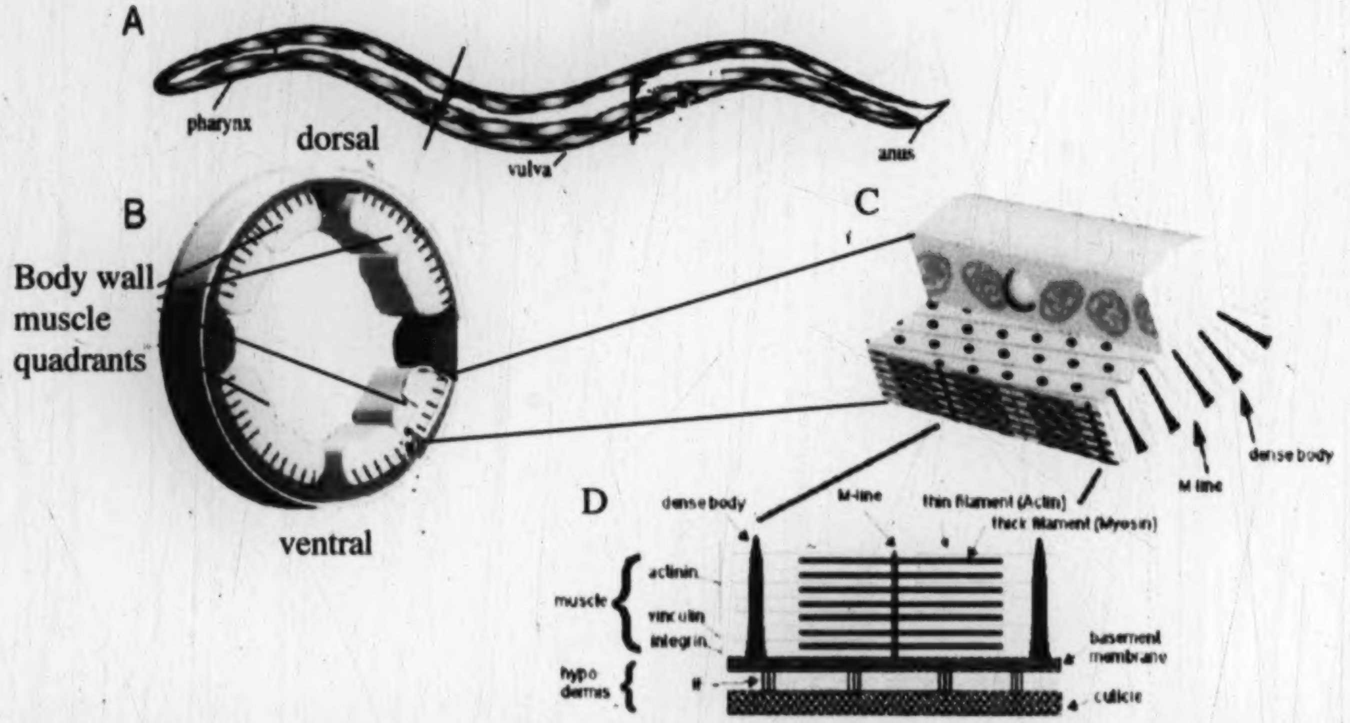

A
pharynx
dorsal
vulva
anus
B
Body wall muscle quadrants
ventral
C
dense body
M line
D
dense body
M-line
thin filament (Actin)
thick filament (Myosin)
muscle
actinin
vinculin
integrin
hypo dermis
basement membrane
cuticle

**Figure 1.1: Body wall muscle structure in *C. elegans*.** In (A) the body wall muscle cells in two of the quadrants are shown. Head is to the left, tail is to the right. (B) is a transverse section taken at the midbody (line and arrow shown in (A)) to show the four muscle quadrants. (C) is a blowup of part of a single cell from one of the four quadrants. (D) shows a single muscle sarcomere (For more detailed view see Figure 1.2) Adapted from Moerman et al., (1997).

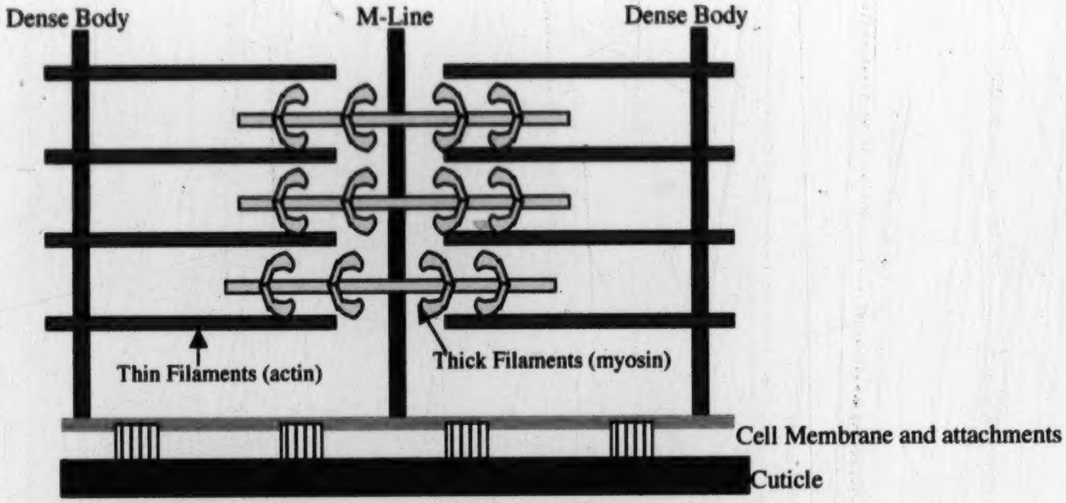

Dense Body
M-Line
Dense Body
Thin Filaments (actin)
Thick Filaments (myosin)
Cell Membrane and attachments
Cuticle

**Figure 1.2: Schematic model of the body-wall myofilament lattice in *C. elegans*.** Contractile force is generated by myosin heads (thick filaments) interacting with and pulling on adjacent thin filaments thereby pulling dense bodies closer together. Transfer of force to the outside of the animal is obtained through a series of attachments to the cuticle. Adapted from Moerman and Fire (1997)

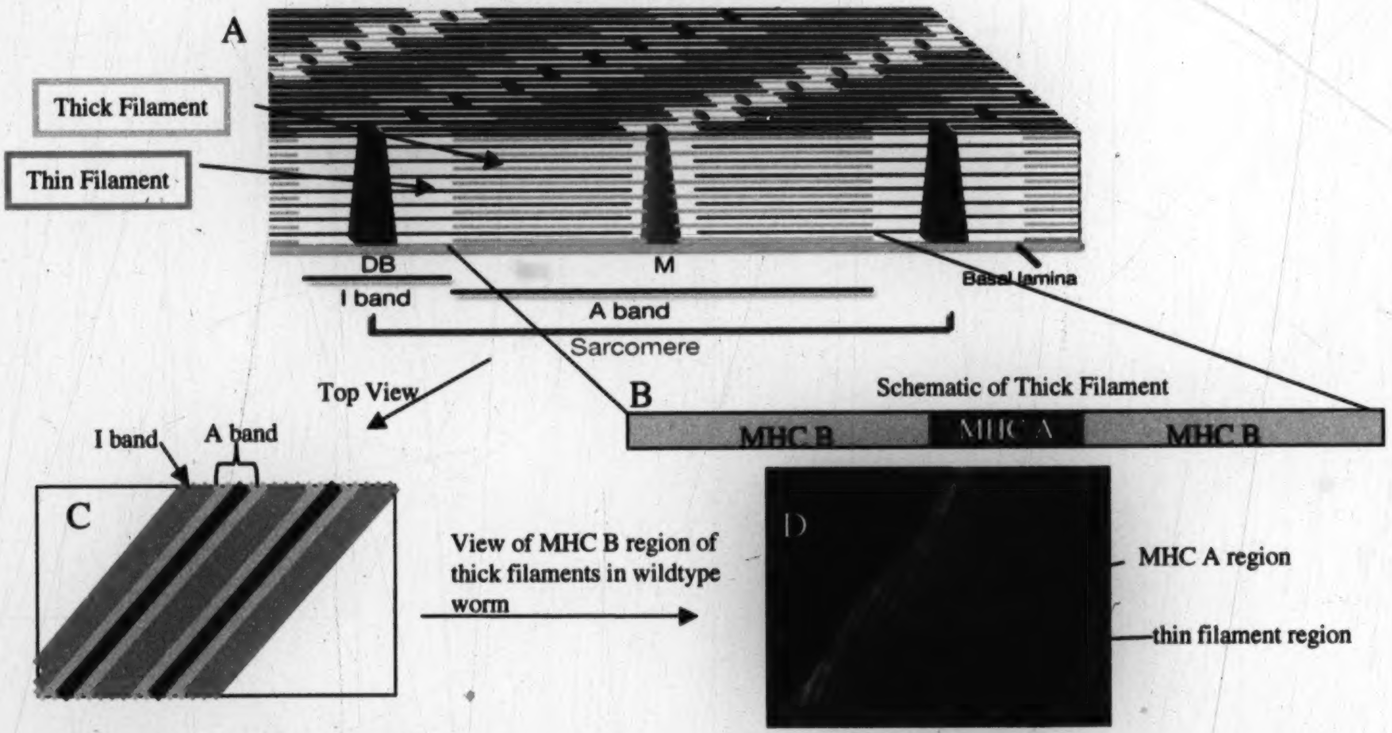

A
Thick Filament
Thin Filament
DB
M
Basal lamina
I band
A band
Sarcomere
Top View
B
Schematic of Thick Filament
MHC B
MHC A
MHC B
I band
A band
C
View of MHC B region of thick filaments in wildtype worm
D
MHC A region
thin filament region

**Figure 1.3: Closer Examination of the thick filament.** (A) indicates how MHC B is incorporated into the sarcomere (DB=dense body, M=M line). Adapted from Worm atlas (http://www.wormatlas.org/handbook/mesodermal.htm/musclepartII.htm) (B) diagrams how the two isoforms of myosin are assembled into the thick filament. See also Figure 1.6 for the assembled thick filament. (C) shows a schematic top view of A and (D) shows immunostaining with an antibody against the MHC B isoform to show how the thick filament looks in wild-type worms. Note that the central MHC A region is unstained as well as the thin filament region that is found separating each thick filament.

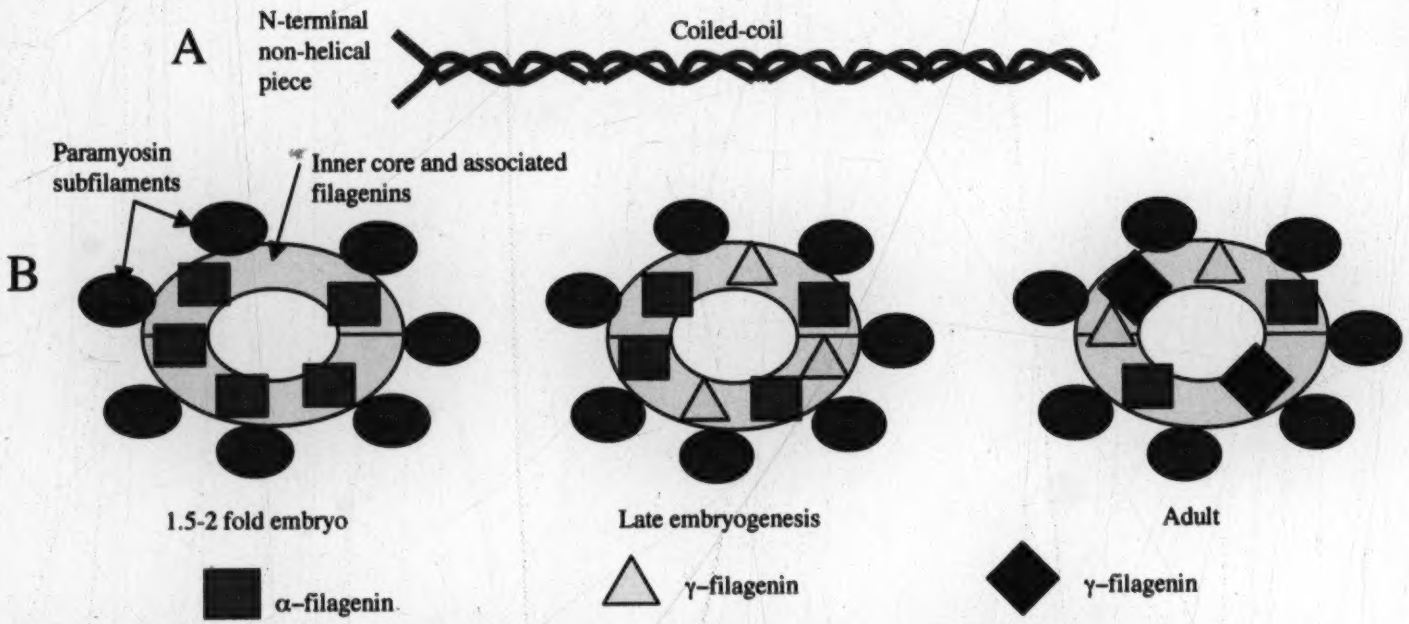

A
N-terminal non-helical piece
Coiled-coil
B
Paramyosin subfilaments
Inner core and associated filagenins
1.5-2 fold embryo
Late embryogenesis
Adult
α–filagenin
γ–filagenin
γ–filagenin

**Figure 1.4: Diagram of the paramyosin containing core of the thick filament.** (A) Structure of paramyosin, which consists of two strands arranged in a coiled-coil with potential phosphorylation sites at the non-helical N-terminus. (B) Cross section of the rigid model of the core structure of *C. elegans* thick filaments (Epstein et al., 1995; Liu et al., 2000). The outer layer is composed of seven paramyosin subfilaments. These subfilaments are cross-linked by a series of internal proteins, the filagenins, which are differentially expressed during muscle development. α–filagenin is exclusively expressed in the nascent linear structure. α–filagenin and γ–filagenin are expressed in the short embryonic thick filament core, whereas all three (α–filagenin, γ–filagenin, and β–filagenin) are expressed in the intermediate larval and long adult thick filament. This differential temporal expression is believed to help control the length of the developing thick filament, and MHC B is elongated using the core as a template. See section 1.2.2 for details.

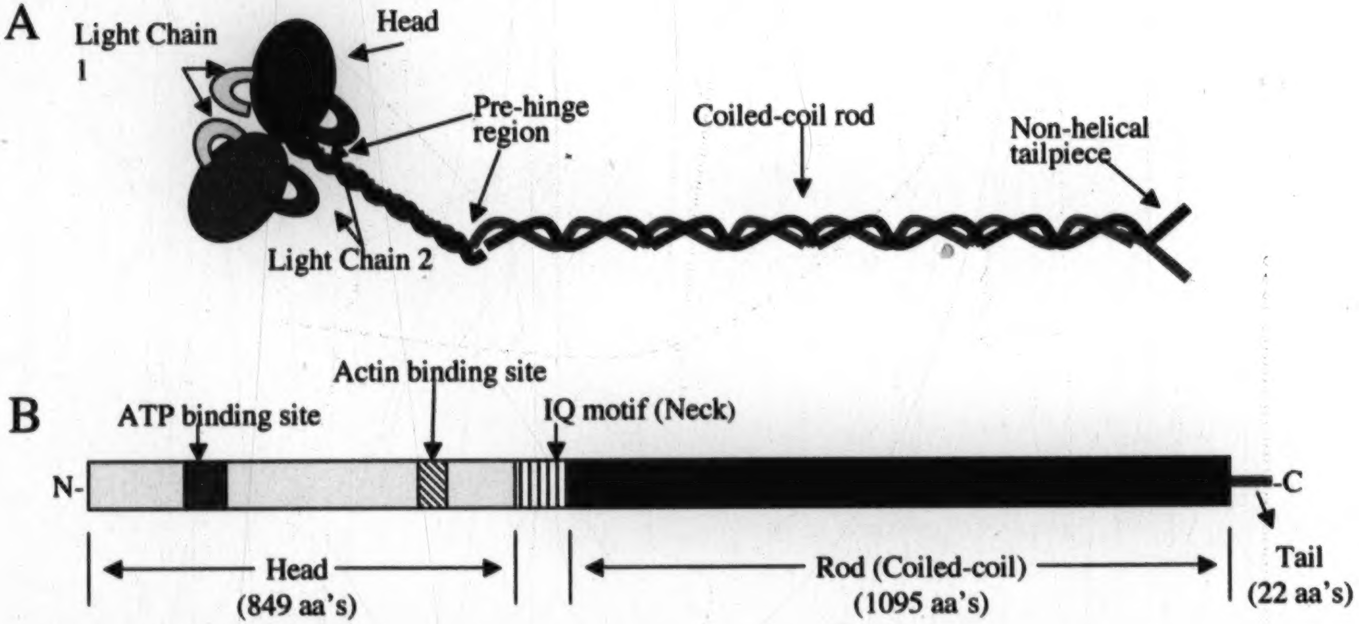

A
Light Chain 1
Head
Pre-hinge region
Coiled-coil rod
Non-helical tailpiece
Light Chain 2
B
Actin binding site
ATP binding site
IQ motif (Neck)
N-
-C
Head
(849 aa's)
Rod (Coiled-coil)
(1095 aa's)
Tail
(22 aa's)

**Figure 1.5: Schematic diagram of conventional Type II myosin found in the body wall muscles of *C. elegans*.** (A) Each molecule is a hexamer composed of two heavy chains and two pairs of light chains. The head domain is the motor domain which interacts with actin and the coiled-coil tail dimerizes and is responsible for assembly of the protein. (B) Schematic diagram of a single heavy chain showing the ATP and actin binding sites in the head, the IQ motif (light chain and calmodulin binding sequence) in the neck domain and the coiled-coil rod. The heavy chain has 1966 amino acids with the breakdown of each region shown (taken from McLachlan, 1984)

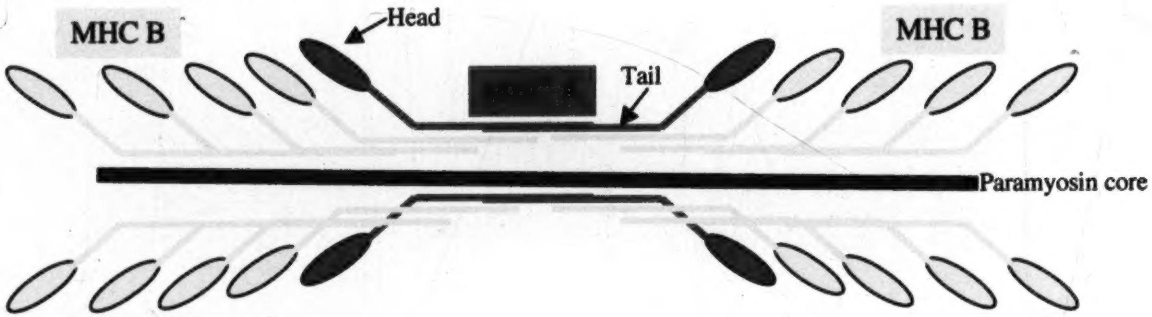


**Figure 1.6: Diagram of the thick filament in *C. elegans* body wall muscles.** The two isoforms of MHC are arranged asymmetrically along the length of the filament. MHC A molecules are packed in an anti-parallel (tail to tail) manner to initiate filament assembly, resulting in a central bare zone devoid of any myosin heads. MHC B molecules are then extended in a parallel manner (head to tail) to the polar ends of the filament to elongate the filament. The central core consists of paramyosin and accessory proteins (See Figure 1.4). Note: for simplicity the myosins are shown as a single molecule, not a hexamer.

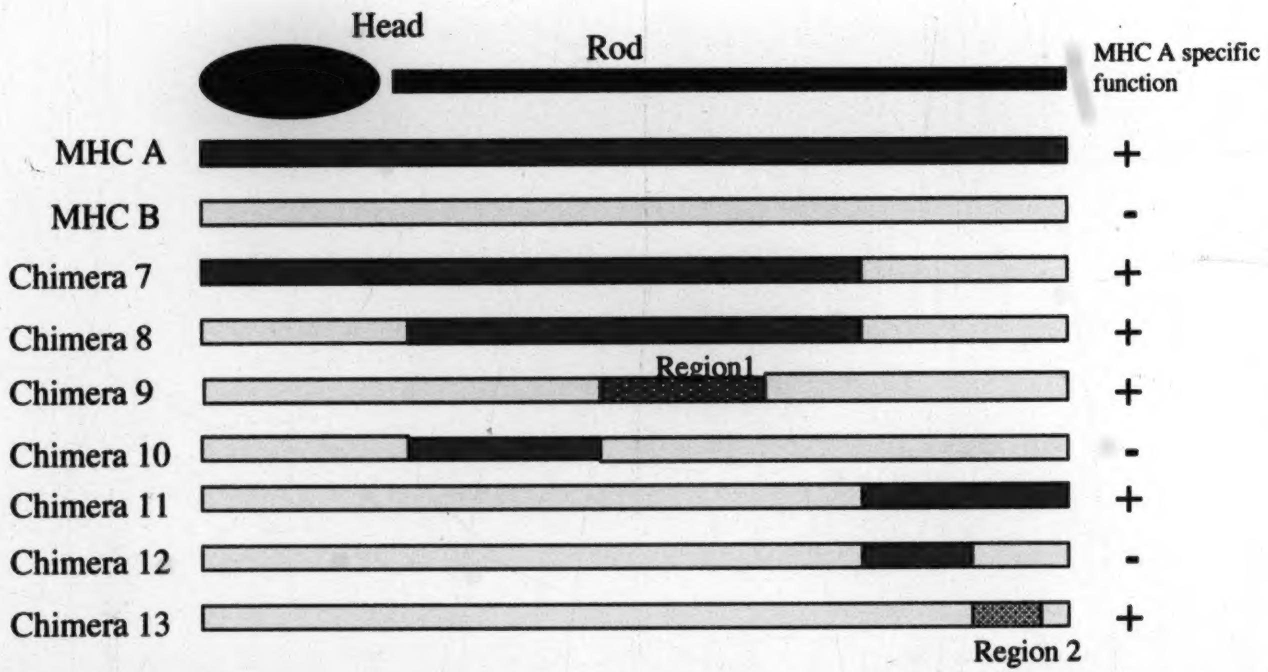

Head
Rod
MHC A specific function
MHC A
+
MHC B
-
Chimera 7
+
Chimera 8
+
Chimera 9
Region1
+
Chimera 10
-
Chimera 11
+
Chimera 12
-
Chimera 13
+
Region 2

**Figure 1.7: Molecular chimeras of MHC A and MHC B used to determine MHC A specific function**. Two regions of the MHC A rod are sufficient for MHC A filament initiation as determined by the rescue of a *myo-3* null mutant (Hoppe and Waterston, 1996). Region 1 is 263 amino acids in length in the middle of the rod and Region 2 is 163 residues near the C-terminus of the rod. Dark regions are MHC A sequence and light regions are MHC B sequence.

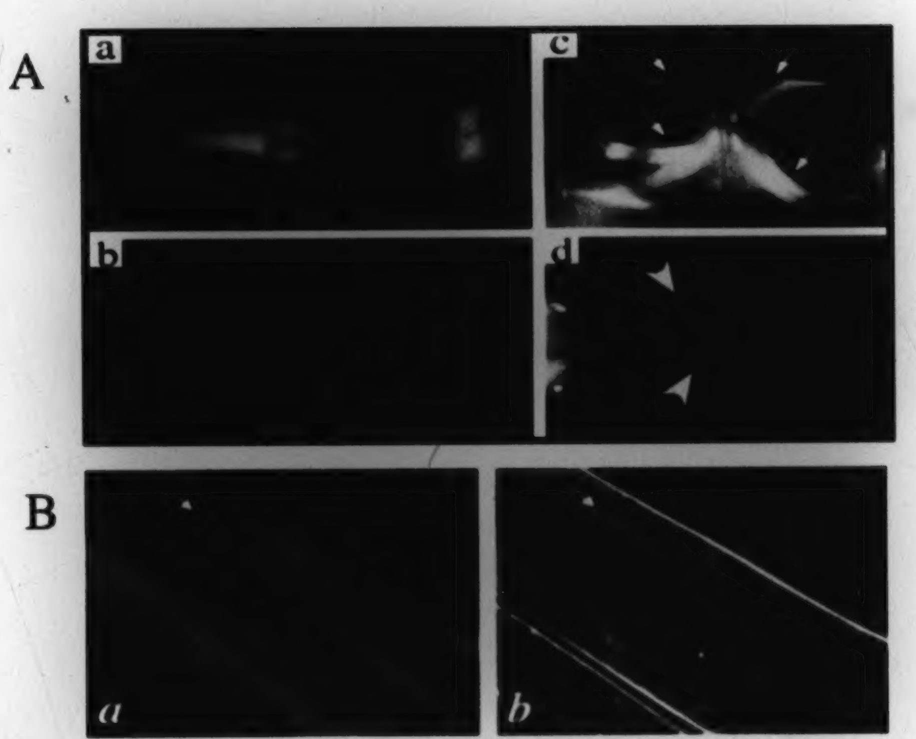


**Figure 1.8 (A)** ***unc-45*****::GFP expression** (Venolia et al., 1999). A GFP reporter driven by the *unc-45* promoter is expressed in pharyngeal muscles (a), body wall muscles (b), vulval muscles (c), and anal muscles (d).
**(B) UNC-45::GFP expression pattern in the body wall muscles** (Ao and Pilgrim, 2000). (a) is the GFP signal associated with the UNC-45::GFP fusion protein. (b) is the same field showing the muscle filament pattern as visualized by polarized light microscopy. The arrows indicate the A bands of the thick filament.

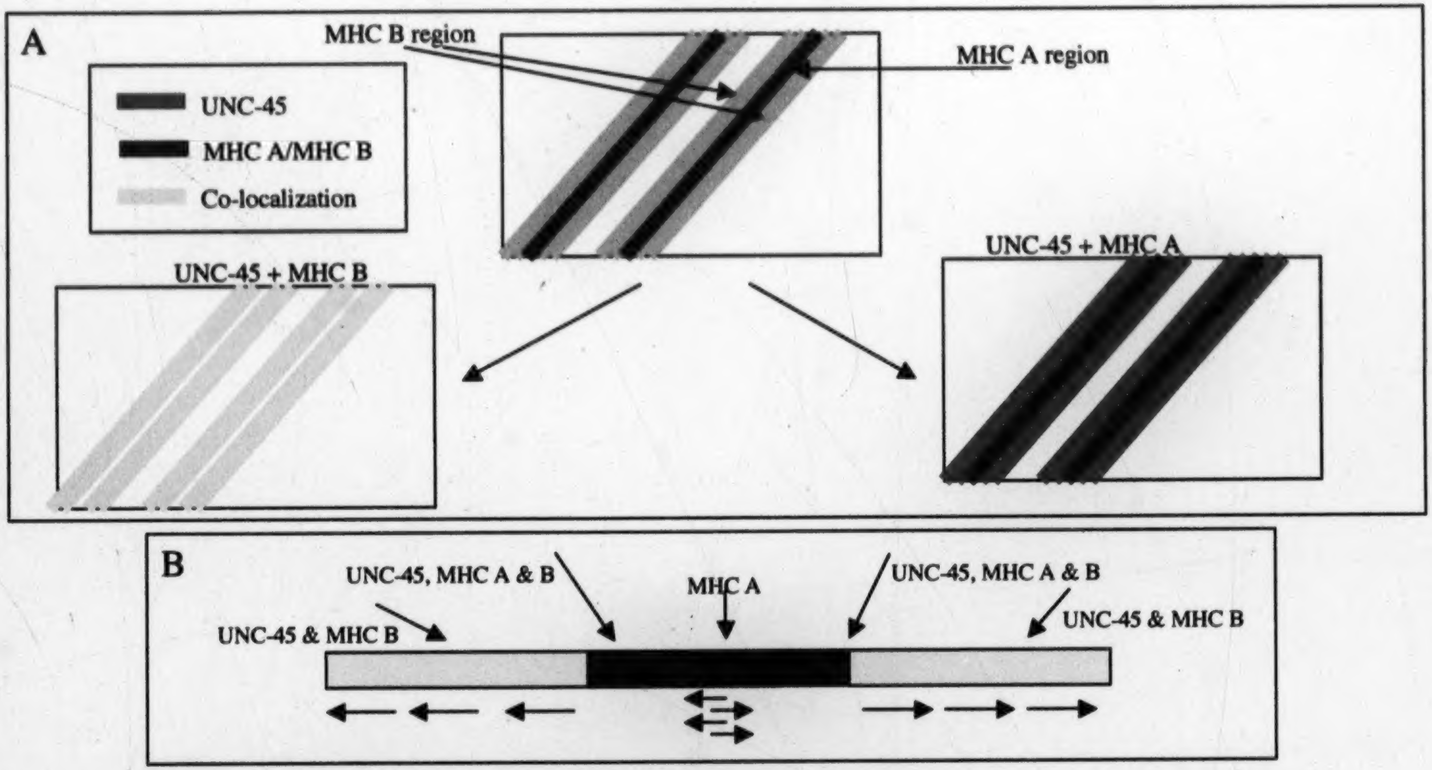

A
MHC B region
MHC A region
UNC-45
MHC A/MHC B
Co-localization
UNC-45 + MHC B
UNC-45 + MHC A
B
UNC-45, MHC A & B
MHC A
UNC-45, MHC A & B
UNC-45 & MHC B
UNC-45 & MHC B

**Figure 1.9: (A) Schematic diagram of UNC-45 immunostaining in thick filaments**. UNC-45 co-localizes with MHC B, but not MHC A, in the body wall muscle thick filaments of *C. elegans*
**(B) Localization of UNC-45 in the thick filament**. UNC-45 is localized to the MHC B conaining outer arms of the thick filament. The central MHC A region contains no UNC-45. All three overlap in a small region between the two.

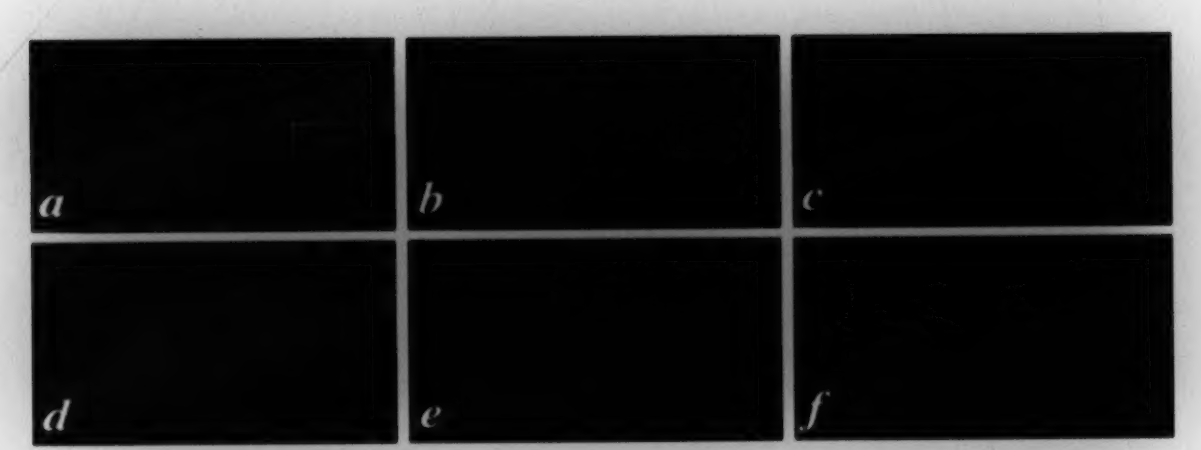


**Figure 1.10: UNC-45 may be added into thick filaments after the MHC isoforms** (Ao and Pilgrim, 2000). a, b, and c are same frame of wild-type L1 larval worm stained with 7N5 (UNC-45)(green), DM 5-8 (MHC B) (red), or double labeled with 7N5 and DM 5-8, respectively. d, e, and f are enlarged for the boxed areas of a, b, and c, respectively, showing one muscle cell. UNC-45 is diffuse in the cytoplasm (d) whereas MHC B has already been assembled into the thick filament near the cell membrane (e).

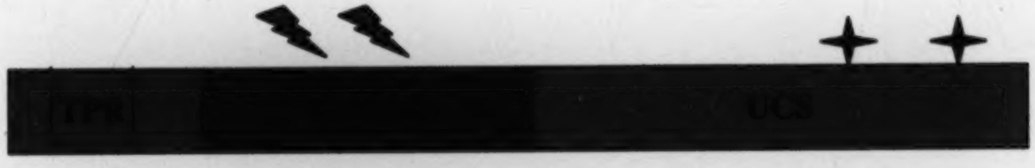


**Figure 1.11: Structure of *C. elegans* UNC-45**. TPR domain is indicated in purple, unique central domain in blue, and the UCS domain in green. The positions of the two null alleles (nonsense mutations) are marked with red lightning bolts and the two temperature sensitive alleles (missense mutations) are marked with blue stars.

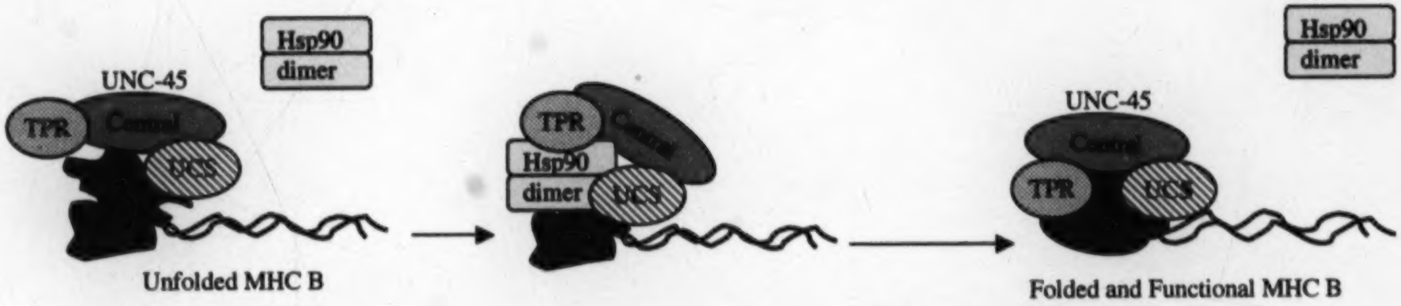

Hsp90
dimer
UNC-45
TPR
Central
UCS
Unfolded MHC B
Hsp90
dimer
TPR
Central
UCS
UNC-45
Hsp90
dimer
Central
TPR
UCS
Folded and Functional MHC B

**Figure 1.12: Model for how UNC-45 may act as a chaperone adapter**. UNC-45 binds Hsp90 through its TPR domain and then binds to myosin through the UCS domain to bring Hsp90 into the vicinity of the myosin head to help it fold. Once folding is complete Hsp90 is released but UNC-45 remains associated with MHC B, potentially to bring Hsp90 back if the myosin head becomes unfolded.

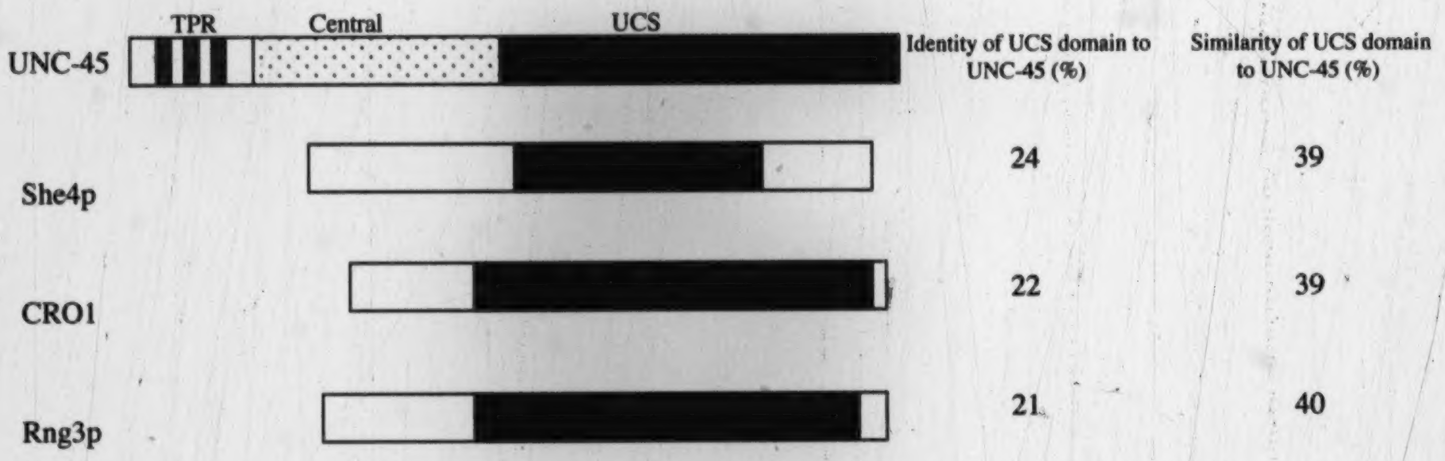

TPR
Central
UCS
UNC-45
Identity of UCS domain to UNC-45 (%)
Similarity of UCS domain to UNC-45 (%)
She4p
24
39
CRO1
22
39
Rng3p
21
40

**Figure 1.13: Alignment of the fungal UCS homologs of UNC-45.** Percent identity and similarity of the UCS domain to UNC-45 is indicated. Black areas indicate region of sequence similarity to UNC-45.

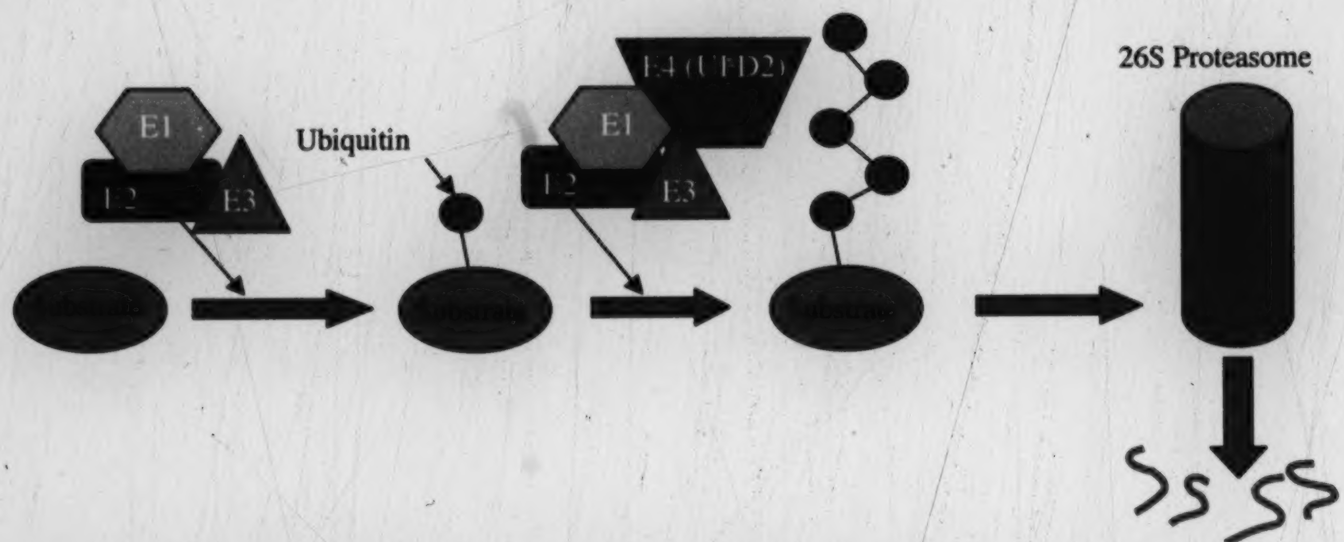

E1
E2
E3
Substrate
Ubiquitin
Substrate
E4 (UFD2)
E1
E2
E3
Substrate
26S Proteasome

**Figure 1.14: The pathway of ubiquitin mediated degradation of a target substrate.** The ubiquitin enzymes, E1, E2, and E3 recognize and attach ubiquitin to the target substrate. Recently identified E4 (UFD-2 in yeast) is needed in some cases as an additional conjugation factor which binds ubiquition moieties of preformed complexes. A multi-ubiquitin chain is assembled on the substrate and degraded by the 26S proteasome.

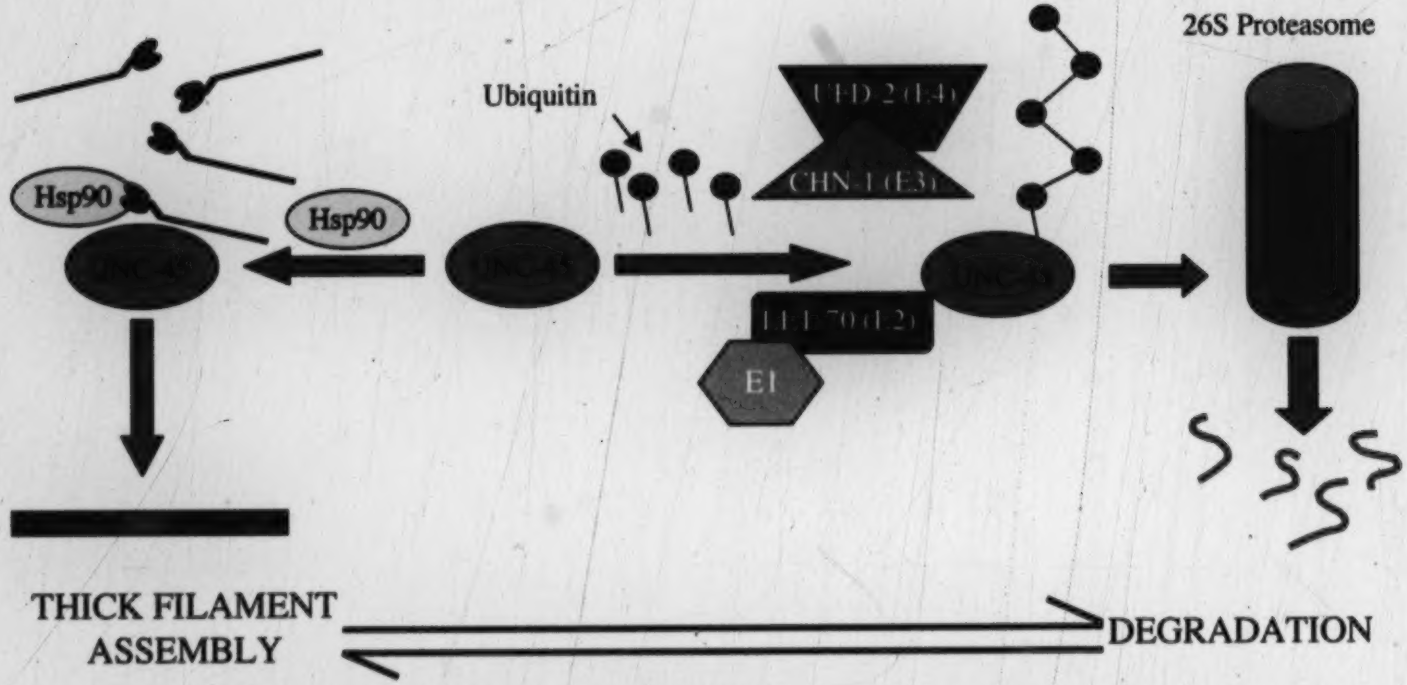

Ubiquitin
26S Proteasome
Hsp90
Hsp90
UNC-45
UNC-45
UNC-45
UFD-2 (E4)
CHN-1 (E3)
LET-70 (E2)
E1
THICK FILAMENT ASSEMBLY
DEGRADATION

**Figure 1.15: Hypothetical model for CHN-1/UFD-2 dependent regulation of UNC-45**. Adapted from Hoppe et al., 2004. UNC-45 is able to bind Hsp90 and myosin to function as a co-chaperone for thick filament assembly. Additionally UNC-45 interacts with CHN-1 and UFD-2 simultaneously to allow for multi-ubiquitination and degradation of itself by the 26S proteasome. UNC-45 appears to be under tight regulation to insure correct assembly of myosin into the thick filaments.

| UCS protein | Organism | Potential Interacting Myosins | Mutant Phenotype | Potential Role of UCS protein |
|---|---|---|---|---|
| CRO1 | Filamentous fungus<br>*P. anserina* | None to date | - failure in syncytial to cellular transition<br>- disorganized actin cytoskeleton | - assemble and regulate myosin to bring it into contact with the actin cytoskeleton |
| She4p | Budding yeast<br>*S. cerevisiae* | Myo5p (Myosin I)<br>Myo4p (Myosin V) | - *ASH1* mRNA mislocalization<br>- endocytosis defects<br>- actin cytoskeleton disorganization | - proper myosin localization to mediate actin-myosin interactions |
| Rng3p | Fission yeast<br>*S. pombe* | Myo2p (non-muscle myosin II<br>- Also Interacts with Swo1p (Hsp90 homolog) | -disrupted actomyosin ring<br>-cytokinesis failure | - assembly and localization of Myo2p and actin into actomyosin ring (cytokinesis)<br>- could be acting as co-chaperone due to interaction with Swo1p |

**Table 1.1: Summary of fungal UCS proteins.** All three interact directly or indirectly with various classes of myosins and are involved in myosin related processes. Adapted from Yu and Bernstein, ( 2003).

## 2. Materials and Methods

### 2.1. Strains and worm maintenance

Unless otherwise stated all worms were grown at 20°C on NGM agar plates seeded with *E. coli* strain OP50 (Brenner, 1974). *unc-54(0)* worms carrying the transgenic arrays expressing the chimeric myosins discussed in the text were kindly provided by Dr. Pamela Hoppe (currently located at Western Michigan University). CB190 [*unc-54(e190)*] and DR176 [*unc-54(e190)I; eDp23 V*] were obtained through the *Caenorhabditis* Genetics Centre. *unc-45(r450)* was from the lab stock collection.

### 2.2. Polyclonal antibody production

For polyclonal antibody production a construct made by a previous student (pDP#WA027) was used for protein production and injection into rabbits (Ao, 2001). WA027 contains a fragment of *unc-45* cDNA corresponding to a 58-residue region from amino acid 18 to 76 of the predicted UNC-45 protein fused in-frame to the glutathione-S-transferase coding region in the expression vector pGEX-2T (Amersham Pharmacia Biotech). I will refer to this as UNC-45(18-76) for the remainder of this thesis. The construct was transformed into *E. coli* BL21 (Stratagene) to allow for IPTG induction of expression. Cells were grown at 37°C in 1 L of 2xYT media for 3 hours, induced by adding 500 μL of I M IPTG, and grown for another 3.5 hours before being pelleted and frozen at –20°C.

For purification, cells were resuspended in 10 ml of PBS and passed through a French Press four times at 1500 psi. Triton X-100 was added to lyse cells at a final concentration of 1% and cells were shaken at room temperature for 30 minutes. Cells were then spun for 10 minutes at 10,000 rpm in a refrigerated centrifuge to pellet insoluble debris. The supernatant was split in half and each aliquot was added to a 50% slurry of Glutathione Sepharose 4B (Amersham Biosciences) and rocked for 30 minutes at room temperature. The slurry+supernatant was centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded. The Sepharose beads were washed three times with 10 ml PBS, pelleting at 1500 rpm after each wash. After washing, 1 ml of Glutathione Elution Buffer (0.154g of reduced glutathione dissolved in 50 ml of 50 mM Tris-HCl pH 8.0) was added to the beads and incubated for 20 minutes with rocking. The beads were then spun at 1500 rpm and the elutant was removed. The elution was performed two more times for a total of 6 ml of purified UNC-45(18-76) protein. The protein was then washed with PBS and concentrated to a final volume of 200 µL using an Amicon Ultra 15 10,000 MWCO Centrifugal Filter Device spun at 3700 rpm (Millipore). The purified protein was then quantitated by comparison to BSA standards run on a 12% SDS-PAGE gel.

Before inoculation of the rabbits a pre-bleed sample was taken from each rabbit to check for cross-reactivity to worm proteins. None of the rabbits showed cross-reactivity to nematode lysates on a Western Blot. For inoculation of the rabbits, the protein was combined with PBS to a total of 750 µl and mixed thoroughly with 750 µl of Freunds's Complete Adjuvant for the first injection. For the remaining boosts, 500 ug of protein were combined with PBS (to a total of 750 µL) and 750 µl of Freund's Incomplete Adjuvant was injected into the rabbits. Rabbits 3E2 and 3E5 were injected five times before exsanguinations. Rabbits 4E1 and 4E5 were injected four times before exsanguinations. 3E2 and 3E5 sera were stored at –80°C and at 4°C. 4E1 and 4E5 sera were stored at –80°C, 4°C and –20°C mixed with 50% glycerol. 7N5 serum was made by a previous graduate student (WA027 injected rabbits) and had been stored at –80°C (Ao, 2001).

### 2.3. Testing antisera for reactivity against UNC-45

Antisera were tested for reactivity against purified recombinant GST::UNC-45(18-76) protein. Protein was run on a 12% SDS polyacrylamide gel and blotted onto nitrocellulose. The membrane was blocked with 5% skim milk powder in TBS-T and then probed with dilutions of immune serum ranging from 1/50 to 1/500 for 2 hours. Detection was carried out using 1/10000 anti-rabbit HRP conjugated secondary antibody (Amersham) for 1 hour and Supersignal West Pico Chemiluminescent Substrate (Pierce) as per the manufacturer's instructions.

All antisera were tested for immunoreactivity against wild-type *C. elegans* (N2 strain) lysate. To prepare the lysate, two 6 cm plates of worms, just clearing of bacteria, were suspended in worm lysis buffer (50 mM ethanolamine, 5 mM DTT, 2 mM EDTA, 1 mM PMSF), microwaved at high power for 25 seconds, and boiled for 5 minutes after adding an equal volume of 2x sample buffer. Samples were run through a 26-gauge needle and pelleted at top speed for 3 minutes in a microfuge. The supernatant was removed and used for imunoblotting as described above.

### 2.4. Antibody purification

Purification was performed by chromatography using an antigen affinity column. Protein was purified from bacteria cells carrying GST::UNC-45(18-76) as described above and 5 mg of protein was exchanged 3 X with 15 mL of coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl pH 8.3) using an Amicon Ultra 15 10,000 MWCO Centrifugal Filter Device spun at 3700 rpm (Millipore). 0.33g of CNBr Activated Sepharose Fast Flow (Amersham) were prepared according to manufacturer's instructions and the protein was added to the beads and incubated at room temperature for 2 hours with gentle rocking. After coupling, the beads were washed once with 10 ml of blocking buffer (0.2M glycine, 0.1M $NaHCO_3$, 0.5M NaCl pH 8.0) and then suspended in 5 ml of blocking buffer and incubated at room temperature for 2 hours with gentle rocking. The supernatant was then removed and the beads were washed 5 times with a cycle of 7 ml coupling buffer/7 ml blocking buffer. Beads were then washed 5 times with 5 ml coupling buffer, 2 times with PBS and then stored at 4°C in PBS. An antigen affinity column was also made with the cleavage product (GST protein and UNC-45(18-76) protein) from the thrombin cleavage reaction (see Section 2.6).

The antibodies were purified by incubating 1 ml of serum (diluted with 4 ml of PBS) with the GST::UNC-45(18-76) conjugated Sepharose. Binding was allowed to proceed overnight at 4°C with gentle rocking. After binding, the beads were washed 3 times for 5 minutes each with 10 ml PBS at room temperature. The washed beads were then transferred to a column and the PBS was allowed to drain. Antibody was eluted from the column in five 1 ml fractions of glycine elution buffer (0.1M glycine pH 2.4). Immediately upon collection fractions were neutralized by adding 90 µl of 1M Tris-HCl (pH 8.8) and 20 µl of 5M NaCl. Western blotting was used to test the purified antibody for reactivity against GST::UNC-45(18-76). After confirmation of reactivity, the five elutions were pooled and concentrated with PBS to a final volume of 200 µl in an Amicon Ultra 15 10,000 MWCO Centrifugal Filter Device spun at 3700 rpm (Millipore). The antibody was then stored at 4°C. This procedure was carried out for sera from rabbits 7N5, 3E2, 3E5, 4E1, and 4E5.

### 2.5. Removal of GST

Affinity strip purification was used to remove any GST antibodies present in the purified polyclonal UNC-45(18-76) antibodies. 200 µl of GST protein was loaded on a 1.5mm SDS polyacrylamide gel using a preparative comb. Following electrophoresis, protein was transferred to a nitrocellulose membrane that was stained with Ponceau S to allow visualization and excision of the GST protein band. The protein strip was washed in water and then blocked in 3% BSA (in PBS) overnight. The strip was then washed twice with water and incubated with 300 µl of purified 3E2 antibody and 2 ml of PBS overnight. The liquid was then removed and concentrated as described above. This procedure was completed for sera from rabbits 7N5, 3E5, 4E1, and 4E5.

### 2.6. Cleavage of UNC-45 from GST

The pGEX-2T vector used to express the GST::UNC-45(18-76) fusion protein (Amersham Pharmacia Biotech) contains a thrombin proteolytic site that can be used to cleave the GST portion from the fusion. Cleavage was performed to isolate the UNC-45(18-76) protein fragment for more stringent purification of an UNC-45 antibody. 1 mg of purified GST::UNC-45(18-76) was added to washed thrombin-agarose resin

(THROMBIN CleanCleave KIT (Sigma)) and incubated for 6 hours as per manufacturer's instructions. The mixture was centrifuged for 5 minutes at 500xg and the supernatant, containing the cleaved products, was transferred to a new tube. To isolate the UNC-45(18-76) fragment (6kDa) the cleavage product was added to 0.5 ml of washed Glutathione Sepharose 4B (Amersham Biosciences) beads and incubated overnight at 4°C with gentle rocking. Beads were spun down at 1500 rpm and supernatant was removed (UNC-45(18-76) only fragment) and concentrated as described above. The concentrated protein was then run on a 15% preparative SDS polyacrylamide gel and transferred to nitrocellulose to make a strip with which to purify antisera. The UNC-45(18-76) fragment was not detected following the transfer to nitrocellulose, possibly due to its small size.

### 2.7. Monoclonal antibody production

For monoclonal antibody production GST::UNC-45(18-76) protein was used to induce an immune response in mice. For the first attempt three mice were injected with 25 μg of protein diluted in PBS and added to an equal volume of Freund's Complete Adjuvant. Four boosts, each two weeks apart, followed with 25 μg of protein diluted in PBS with 750 μl Freunds Incomplete Adjuvant. A final boost was performed with GST::UNC-45(18-76) only protein in PBS. Antisera were tested for reactivity by the use of a dot blot. 5 μg of GST::UNC-45(18-76) was dotted onto a nitrocellulose membrane and blocked in 3% BSA overnight. Various dilutions of mouse antisera were used to probe the membrane and incubated for 2 hours. The membrane was washed four times with 5 minute washes of TBS-T and then detection was carried out using 1/10000 anti-mouse HRP conjugated secondary antibody (Amersham) for 1 hour and Supersignal West Pico Chemiluminescent Substrate (Pierce) as per the manufacturer's instructions. For the second set of mice the same procedure was followed as described above but the mouse with the best response died during final boost of antigen. A terminal bleed was then taken and a polyclonal antibody was made from serum (as described above).

### *2.7.1. Myeloma fusion*

Fusion was performed with the generous help of Dr. Manijeh Pasdar. A week before fusion was to take place SP20 myeloma cells were thawed from liquid nitrogen and re-grown. A day before the fusion cells were split into fresh RPMI medium supplemented with 10% FBS at a concentration of $5 \times 10^5$ cells/mL. On the morning of the fusion SP20 myeloma cells were collected in RPMI media (without serum), washed once, and resuspended in 10 mL of RPMI media. The mouse was sacrificed and the spleen was removed and placed in a petri dish with RPMI media without serum. Using a needle, a small amount of RPMI was injected into the spleen. The spleen was teased apart using 19 gauge needles until most of the cells had been released and spleen was torn into very small pieces. Cell clumps and tissue were broken apart and the clumps of tissue were removed. The cells were transferred to a 50 mL tube and centrifuged for 5 minutes at 400xg, resuspended in 10 mL of RPMI media and centrifuged again, and another 10 mL of RPMI was added. The spleen and the myeloma cells were mixed together in a 15 mL tube and centrifuged for 5 minutes at 400xg. The medium was removed and 0.22 mL of 30% PEG was added drop-wise to the cells, which were then mixed very gently by flicking and centrifuged for 3 minutes at 300xg. The PEG was removed, 5 mL of RPMI was added and cells were gently resuspended, spun again for 3 minutes and resuspended in 5 mL of complete media (RPMI + 10% FBS, 5 mL PSK, 5 mL sodium pyruvate, 5 mL non essential amino acids, and 1X HAT). Cells were added to 250 mL of complete media and 100 µL of cells were dispensed into 20 96 well plates and placed in 37°C, 5% $CO_2$ incubator. Cells were fed every second day by removing 50 µL of media and replacing with 50 µL of fresh complete media.

### 2.7.2. *Screening and expanding positives*

Antigen coated plates were made to screen positives by ELISA. A solution of 2 µg/mL GST::UNC-45(18-76) (in 50 mM carbonate buffer pH 9.0) was made and 50 uL of diluted antigen were added to each well and incubated overnight at 4 °C to coat the plates with GST::UNC-45(18-76) protein. Plates were washed twice with PBS, blocked with 3% BSA overnight at 4°C, and then washed twice with PBS. 50 µL of the monoclonal supernatants along with a 1/5000 dilution of positive control (GST::UNC-45(18-76) polyclonal antibody) and PBS for negative control were added to individual wells for each screening. Plates were then incubated for 2 hours at room temperature, washed 3 times in PBS, and a 1/500 dilution of secondary antibody was added (anti-mouse AP (Molecular Probes) and anti-rabbit AP (Biorad) for 1 hour. Plates were washed three times with PBS and positives were detected by adding 100 µL of Detection Solution (2 tablets of Sigma 104 Phosphate Substrate dissolved in 10 mL of DEA (0.1M Diethanolamine) to each well and incubating in the dark for 30 minutes. Reaction was stopped by adding 100 µL 0.4M NaOH and results were quantitated with an ELISA plate reader. Positives identified by ELISA were transferred to a 24 well plate in 500 µL of complete RPMI and allowed to grow. Finally, they were transferred to a T-25 tissue culture flask and single cell cloned through limiting dilution in a 96 well plate. Limiting dilution was done by diluting original clone to 500 cells/100 µL and placing them in the first well of a 96 well plate. Remaining wells already contained 50 µL of complete RPMI media and cells in first well were diluted 1 in 2 down the left hand row of the plate and 1 in 2 across the plate adding 50 µL of cells to each well. Single cell clones were allowed to grow and screened by ELISA. If positive they were expanded further into 24 well plates and T-25 flasks and supernatants were used for Western analysis and immunostaining.

## 2.8. Fixation of adult *C. elegans*

### *2.8.1. Finney Ruvkun fixation (*Adapted from Finney and Ruvkun (1990)

Worms were washed off plates and rinsed with M9 for half an hour (all centrifugation steps were done at 2000 rpm). They were added to fixation buffer (1 mL of 2X MRWB [(160mM KCl, 40mM NaCl, 20mM EGTA, 10mM spermidine, 30mM Pipes, pH 7.4, and 50% methanol)], 200 µL of 10% paraformaldehyde, 800 µL water) and freeze thawed three times using a dry ice/ethanol and warm running tap water and then rocked at 4°C for 1 hour. Worms were washed twice with Tris-Triton Buffer (100mM Tris Cl pH 7.4, 1% Triton X-100, 1 mM EDTA) and incubated in 1% β-mercaptoethanol (in Tris-Triton Buffer) for 2 hours at 37°C with gentle rocking. Afterwards worms were rinsed once in 1X$BO_3$ (made from 100X $BO_3$ buffer [(1M $H_3BO_3$, 0.5M NaOH)] and incubated with rocking in 0.5M DTT in 1X $BO_3$ for 15 minutes, rinsed again with 1X $BO_3$, and incubated with rocking in 3% $H_2O_2$ in 1X $BO_3$ for 15 minutes. After a final rinse with 1X $BO_3$, worms were incubated in AbB (0.1%

BSA, 0.5% Triton X-100, 0.05% sodium azide, 1mM EDTA in PBS) for 30 minutes and stored in AbA (same as AbB but with 1% BSA) at 4°C until staining.

### *2.8.2.* *Bouins fixative* (Performed as described in Nonet et al., (1997)

Worms were washed off the plate and rinsed with M9 for half an hour and most M9 (save 50 µL) was removed. A mix of 400 µL Bouins (15 mL saturated picric acid, 5 mL formalin, and 1 mL glacial acetic acid), 400 µL methanol, and 10 µL β-mercaptoethanol was added to the worms and rocked for 30 minutes at room temperature. The tube was quickly frozen in liquid $N_2$ and thawed under running hot water (until the liquid melted but before it warmed up to room temperature) and rocked another 30 minutes at room temperature. The worms were centrifuged (2000rpm) from fixative and 1.4 mL of BTB was added [(1X Borate Buffer (made from 100X Borate Buffer- 1.0M $H_3BO_3$, 0.5M NaOH), 0.5% Triton X-100, 2% β-mercaptoethanol)], rocked a few times, centrifuged, and aspirated. Two more washes were done and the worms were suspended in 1 mL of BTB and rocked for 1 hour at room temperature. The worms were washed again with 1 mL of BTB and incubated 2-3 more hours (depending on the rate picric acid destains). Worms were then washed with BT (BTB without β-mercaptoethanol) once, AbA twice, and then incubated with AbA for 30 minutes. Worms were stored at 4°C until staining.

### **2.9. Immunostaining**

For immunostaining, worms were incubated in primary antibodies (UNC-45(18-76) (4E1cleaved) 1/250, MHC A (5-6) 1/250, and MHC B (5-8) 1/250, overnight at 4°C in AbA, (MHC A and MHC B monoclonal antibodies were kindly provided by Dr. David

Miller (Miller et al., 1986)). In the morning they were washed for 2 hours in AbB, and incubated with secondary antibodies (1/1500 anti-mouse Alexa 488, 546, 1/1500 anti-rabbit Alexa 488, 546) (Molecular Probes) for two hours. Finally, worms were washed in AbB for two hours and mixed with equal volumes of mounting media (2 mg n-propyl gallate in 70 µL glycerol, 30 µL 100 mM Tris Cl pH 9.5) and mounted on 2% agarose pads for viewing.

### 2.10. Microscopy

Immuno-flourescence images were taken using a Zeiss Axioskop 2 (Carl Zeiss) using the 63X lens (N.A. 1.4) or a confocal microscope and processed using Adobe Photoshop 7.0.

### 2.11. Double mutant creation and confirmation

The transgenic double mutants *unc-45(r450);unc-54(e190)+array* were constructed by crossing temperature sensitive *unc-45(r450)* homozygous males at the permissive temperature (16°C) to hermaphrodites carrying the transgenic array and homozygous for *unc-54(e190) (unc-54(0)* (Figure 2.1). Individual F1 cross progeny were placed one per plate on separate plates, and grown at the permissive temperature. From one of those plates, 40 F2 hermaphrodites were singled out on separate plates, and grown at the permissive temperature. The F3 generation was then scored for *unc-54(e190)* homozygotes by looking for a plate of animals in which every worm that had lost the transgenic array (GFP(-)) were paralyzed. These plates of worms were then switched to the restrictive temperature (20°C) and scored for the *unc-45* phenotype (paralysis), indicating homozygotes for the *r450 ts* allele. These were identified by 100% of the progeny becoming paralyzed regardless of whether the rescuing array (GFP) is present. However, if the chimeric myosin does not require UNC-45 to assemble then the worms containing the array (GFP) would not become paralyzed. Two separate tests were done to confirm that these worms contained the *unc-45(r450)* mutation.

For the first confirmation, the putative *unc-54(e190);unc45(r450ts) + array* worm was crossed to an *unc-45(r450)* male and the F1 progeny that had lost the array (GFP(-)) were scored (Figure 2.2). If 100% of the F1s that had lost the GFP were paralyzed then these worms are double *unc-54(e190);unc-45(r450)* mutants. If the worms were homozygous wild-type for *unc-45* I would expect 100% moving and if they were heterozygous I would have expected 50% moving and 50% paralyzed worms.

For the second confirmation, the putative *unc-54(e190);unc-45(r450ts) - array* (no GFP and therefore paralyzed) worm was crossed to a *him-8* male (wildtype copies of *unc-54* and *unc-45*) (Figure 2.3). Moving F1s (cross-progeny) were singled and the F2s were scored for their unc:wildtype ratio. A single mutant would have 25% unc progeny, while a double mutant would have 44% unc progeny since the two loci are unlinked.

*unc-54(e190);unc-45(r450ts)* worms that passed both confirmation tests were determined to be double mutants in which the chimeric myosin does not need *unc-45* to be present.

### 2.12. Deletion constructs

All constructs originated from the parent plasmid pDP#WA036 that contains full length *unc-45* cDNA driven by the *unc-45* promoter fused in-frame with GFP at the C-terminus (Ao, 2001). The TPRonly construct was made by digesting WA036 with NheI and XmaI to release a 2.4 Kbp fragment containing the unwanted cDNA. An oligo containing an NheI site and XmaI site on each end (upper: 5'CTAGCCGGCGGTGGAAGATCTGGCGGAGGTC3' and lower: 5'GCCGCCACCT TCTAGACCGCCTCCAGGGCC3') was ligated into the cut vector to reanneal the plasmid which now contains the *unc-45* promoter and the TPR domain (first 441bp of the cDNA) fused in-frame with GFP. Proper ligation was confirmed by digestion with BglII, which was incorporated into the oligo. Digestion of the ligated construct produced 1.9 Kbp and 7.4 Kbp fragments. To insure oligo insertion maintained the proper reading frame, sequencing was performed across the insertion site with the use of primer 5'CGACGGGGCCGATGTAAAG3'. TPR/Central construct was made by digesting pWA036 with MluI and XmaI, which releases a 1.2 Kbp fragment containing the unwanted cDNA. An oligo containing an MluI and XmaI site on each end (upper: 5'CGCGTGTGGCGGAGGTGGAAGATCTGGCGGAGGTC3' and lower: 5'ACACCG CCTCCACCTTCTAGACCGCCTCCAGGGCC3') was ligated into the cut vector to reanneal the plasmid, which now contains the *unc-45* promoter and the TPR and Central domain (1.6 Kbp of cDNA) fused in-frame with GFP. Proper ligation was confirmed by digestion with BglII, which was incorporated into the oligo. Digestion of the ligated construct produced 3.2 Kbp and 7.3 Kbp fragments. To insure oligo insertion maintained proper reading frame sequencing was performed with the use of primer 5' GGCCGCTTCACAGGATCATT3'. UCSonly construct was made by first digesting pWA036 with a partial PstI digest (0.2 µL PstI for 2 minutes at 37°C) that will linearize pWA036. Next an MluI digest was performed and the resulting products were run on a 0.7% agarose gel to select for the 10 Kbp plasmid that was cut at the correct PstI site, which released the TPR and Central domain of UNC-45. Next an oligo was ligated into the vector containing a PstI site on the 5'end and an MluI site on the 3' end (upper: 5'GGCGGTGGGGAAGATCTGGCGGAGGTTA3' and lower: 5'ACGTCCGCCACCT TCAAGACCGCCTCCAATGCGC3') to anneal the *unc-45* promoter to the UCS domain fused in frame to GFP. Proper ligation was confirmed by digestion with BglII (incorporated into the oligo), which released 1.5 Kbp and 8.6 Kbp fragments. To insure oligo insertion maintained proper reading frame sequencing was performed with the use of primer 5'GCGAATTGCGCGAGTTTTCA3'. Due to technical difficulties the construct containing the Central/UCS domain of UNC-45 was not completed. The size difference between full length WA036 (11.7 Kbp) and WA036 minus the TPR domain (11.4 Kbp) was too small to allow them to be resolved from one another.

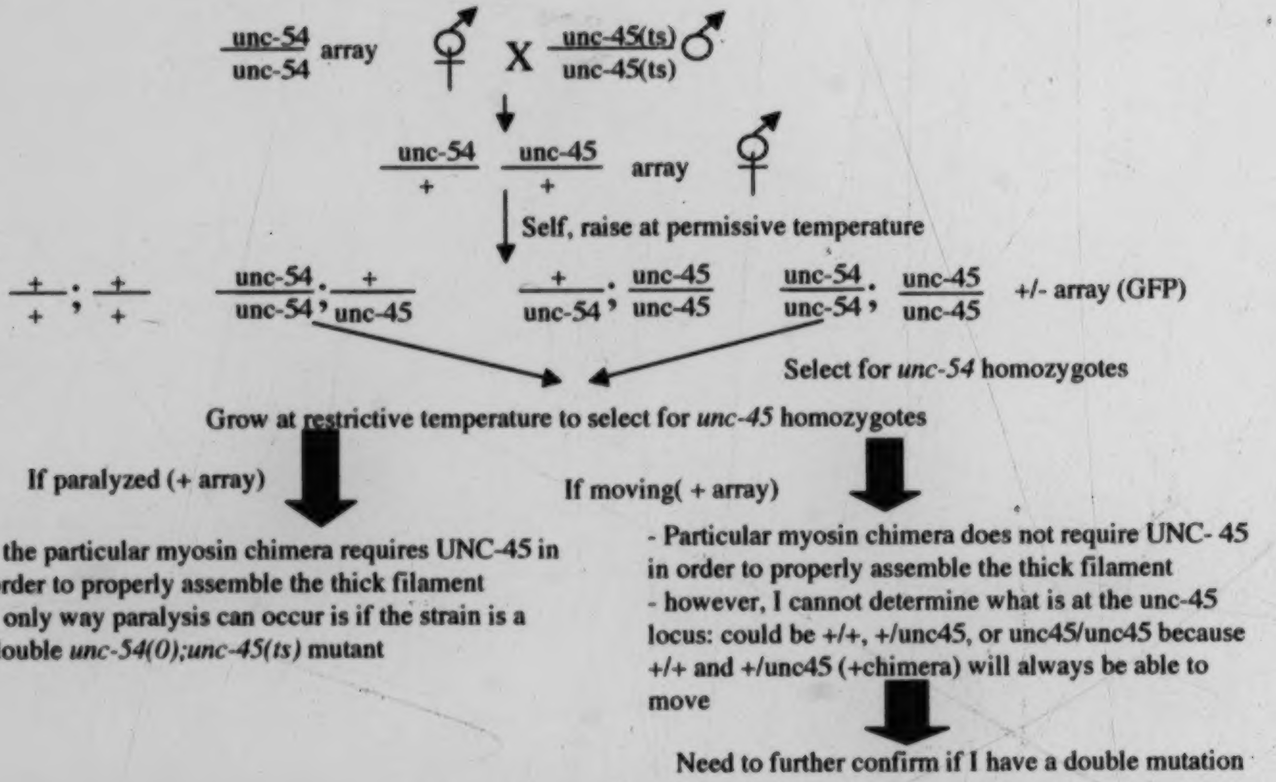

unc-54 / unc-54 array ⚥ X unc-45(ts) / unc-45(ts) ♂
unc-54 / + unc-45 / + array ⚥
Self, raise at permissive temperature
+ / + ; + / +
unc-54 / unc-54 ; + / unc-45
+ / unc-54 ; unc-45 / unc-45
unc-54 / unc-54 ; unc-45 / unc-45
+/- array (GFP)
Select for *unc-54* homozygotes
Grow at restrictive temperature to select for *unc-45* homozygotes
If paralyzed (+ array)
If moving( + array)
- the particular myosin chimera requires UNC-45 in order to properly assemble the thick filament
- only way paralysis can occur is if the strain is a double *unc-54(0);unc-45(ts)* mutant
- Particular myosin chimera does not require UNC- 45 in order to properly assemble the thick filament
- however, I cannot determine what is at the unc-45 locus: could be +/+, +/unc45, or unc45/unc45 because +/+ and +/unc45 (+chimera) will always be able to move
Need to further confirm if I have a double mutation

**Figure 2.1: Mating scheme used to obtain double mutants.** The above crosses were done to obtain an *unc-54(0);unc-45(ts)* mutant for each of the strains carrying the chimeric myosin in an extrachromosomal array. If double mutant was not paralyzed further tests had to be performed to confirm the presence of the two mutations.

$$\frac{\text{unc-54}}{\text{unc-54}}\ \frac{?}{?}\ ⚥ + \text{array} \quad \times \quad \frac{\text{unc-45}}{\text{unc-45}}\ ♂$$

$$\Big\downarrow \text{ examine F1s that have lost transgenic array (e.g. GFP(-))}$$

If unc-45 locus in parent hermaphrodite is +/+: F1 are all $\frac{+}{\text{unc-45}}$ = 100% moving

If unc-45 locus in parent hermaphrodite is +/unc-45: F1 are either : $\frac{+}{\text{unc-45}}$ = 50% moving

OR $\frac{\text{unc-45}}{\text{unc-45}}$ = 50% paralyzed

If unc-45 locus in parent hermaphrodite is unc-45/unc-45: F1 are all $\frac{\text{unc-45}}{\text{unc-45}}$ $\boxed{\textbf{= 100\% paralyzed}}$

**Figure 2.2: First Crossing Scheme to Confirm Double Mutant.** The cross above was done to determine whether I had obtained a double mutant capable of movement in an *unc-45(ts)* background.

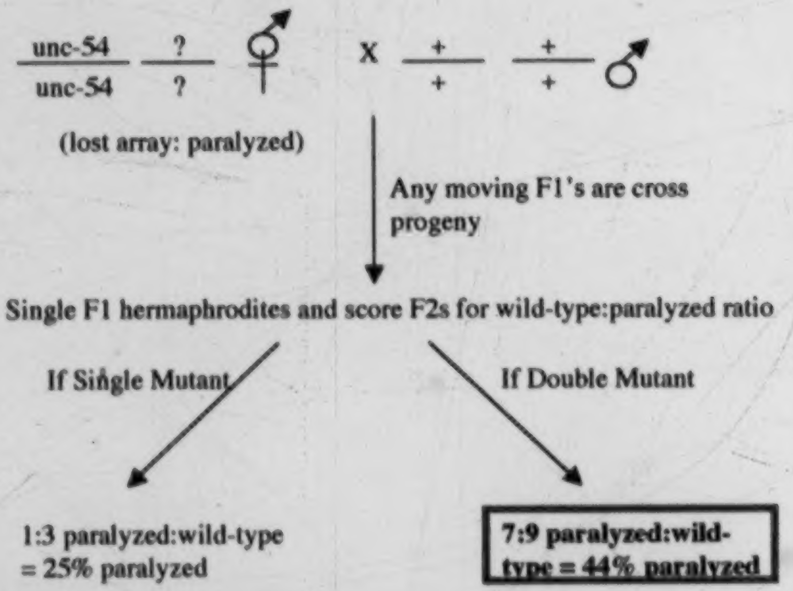

unc-54 / unc-54 ? / ?
x + / + + / +
(lost array: paralyzed)
Any moving F1's are cross progeny
Single F1 hermaphrodites and score F2s for wild-type:paralyzed ratio
If Single Mutant
If Double Mutant
1:3 paralyzed:wild-type = 25% paralyzed
7:9 paralyzed:wild-type = 44% paralyzed

**Figure 2.3: Second crossing scheme to confirm double mutant.** The following cross was performed on the double mutants isolated from the cross in Figure 2.2. See Table 3.1 for results.

# 3. Results

## 3.1. Repurifying old antibody and serum

Wanyuan Ao, a previous student in our lab, had made an antibody against a fusion protein containing a 58 amino acid region within the TPR domain of the UNC-45 protein (amino acids 18 to 76), referred to as GST::UNC-45(18-76) (Ao and Pilgrim, 2000). Purified antibody had been stored at 4°C and –20°C as well as a batch of serum (from rabbit 7N5) at –80°C. I attempted immuno-staining using several aliquots of purified antibody and could not get a specific staining pattern. When visualized by fluorescence microscopy I saw nothing but a hazy background when observing UNC-45 staining, whereas the controls (MHC A and MHC B) worked well each time. I attempted to purify the serum, which had been kept frozen at –80°C, using an antigen affinity column bound with the UNC-45(18-76) fragment corresponding to the region the antibody was generated from, creating a new 7N5 antibody. Western analysis showed that the antibody reacts very strongly to GST::UNC-45(18-76) purified protein, as well as a bacterial protein that seems to co-purify. I attempted to use this antibody on immuno-staining and again only saw a faint hazy background signal. To test whether the antibody was reacting to purified protein I performed a competition assay and showed that as the amount of protein mixed with the antibody increased, the signal of the antibody reciprocally decreased confirming the antibody is specific to UNC-45. As another confirmation of specificity, I performed a Western of a timed induction of GST::UNC-45(18-76) in BL-21 cells (Figure 3.1). The antibody signal increased with induction time indicating the antibody reacts to the fusion protein it was raised against. Since I knew the 7N5 antibody was reacting to the fusion protein I decided to optimize the sample fixation conditions.

All previous immunostaining had been done using a modified Finney/Ruvkun procedure (adapted from Finney and Ruvkun, 1990). I also fixed worms using Bouins fixation (Nonet et al., 1997), which did not increase success, as all resulted in background staining with 7N5 and excellent control (MHC A and MHC B) staining. At this point I concluded the serum had lost the ability to recognize UNC-45 during immunostaining so I decided to make a new antibody.

### 3.2. Monoclonal antibody production

Since even at its best the polyclonal antisera Wanyuan used was not optimal, I decided to make a mouse monoclonal antibody against part of UNC-45. I injected three mice with the same protein fragment used to make the 7N5 antibody. Dot blots showed that all three mice mounted an immune response to the GST::UNC-45(18-76) protein, whereas a normal control mouse showed no reactivity (Figure 3.2). The mouse with the strongest response was chosen for the myeloma fusion (performed with the help of Dr. Manijeh Pasdar) and cells were aliquoted into 20, 96 well plates. Successful fusions were screened for production of UNC-45 antibody through the use of ELISA. 5 individual colonies (out of 1920) had a very strong response on ELISA and were expanded to enable single cell cloning. Out of the 5 strong positives only two showed a response on a Western against purified protein (Figure 3.3). These two cell lines, however, had become very sick. The cells were very small and crinkled and appeared to have stopped dividing. I could no longer maintain them but saved the little supernatant remaining. During this time, I also immunized three new mice with the same protein fragment to attempt the monoclonal procedure once more. All three mice showed a very strong response to the protein fragment; however, the mouse I chose for the fusion (M 1-1) died during the final boost of the protein. The remaining two mice seemed to show signs of distress but recovered. The mouse that had died was exsanguinated and serum was kept as a polyclonal antibody. The intent was to perform a hybridoma fusion on mice M 1-2 and M 1-3, however the myeloma cells were not at their optimal concentration, so they had to be combined into only one fusion (M 1-3) (again fusion was performed with the help of Dr. Manijeh Pasdar). Contamination subsequently affected all of the 96 well plates so the procedure was terminated. I had obtained blood from the mice in which the fusion was not performed (M2, M3, M1-1, and M1-2) as well as test bleeds for mice M1 and M1-3 so I created polyclonal antibodies using the same procedure as described for the 7N5 antibody. All of the antibodies gave variable and conflicting immunostaining results. Vulval muscle staining was seen in some worms as well as thick filament staining. Unfortunately, the filament staining was not consistent, with some worms having what looked like an MHC A pattern while others seemed to have the MHC B pattern (Figure 3.4)

### 3.3. Polyclonal antibody production

As I was making the myeloma fusions I had also injected two rabbits (3E2 and 3E5) with GST::UNC-45(18-76) as this process had worked previously (Ao and Pilgrim, 2000). Western analysis of the test bleeds showed a strong reaction to purified protein (3E2 and 3E5) as well as a faint pattern on immuno-staining (3E2 only). I purified antibody from test bleed number 3 of rabbit 3E2 by running the serum over a GST::UNC-45(18-76) antigen affinity column. Immunostaining with this antibody worked very well (Figure 3.5). I saw the expected UNC-45 pattern in the body wall muscles (similar to MHC B pattern) as well as vulval muscle staining. There was no detectable pharynx staining. However, after about a week of use the staining with this antibody decreased to hazy non-specific background. I obtained another test bleed from rabbit 3E2 and re-purified the serum but specific staining was again not obtained. At this point I tested whether the GST fragment of the protein could be interfering, i.e. if the rabbits were mounting a stronger immune response to the GST portion than to the UNC-45 portion of the fusion. The UNC-45 fragment of the protein is only 6 kDa while the GST portion is 27 kDa. This mass difference (75% of the fusion protein is GST) gives the GST fragment a much higher probability of being targeted by the rabbit immune system and may increase the amount of GST antibody being produced, overwhelming UNC-45 antibody production. To isolate the UNC-45 cross-reacting antibody from the serum I took advantage of a thrombin cleavage site located between the UNC-45 fragment and GST in the fusion protein.

Two new rabbits (4E1 and 4E5) were set up with the intention of injecting the UNC-45 only fragment to elicit an UNC-45 specific response (no GST). To do this I had to separate the GST from the GST::UNC-45(18-76) fragment in the cleavage reaction. I incubated the cleavage reaction with GST beads (Amersham) to allow the GST protein fragment to bind to the beads. I took the supernatant and concentrated it, which should only contain the 6 kDa UNC-45(18-76) fragment (Figure 3.6). Unfortunately I could never get enough of it to work with, as it had either degraded or was too small to purify. Since the rabbits were available I injected them with the GST::UNC-45(18-76) to see if I could get a better response. Western analysis of the test bleeds revealed the rabbits were producing antibodies that recognized the GST::UNC-45(18-76) protein and they also showed a faint staining pattern (in immunofluorescense of fixed whole mount worms), although with high background. The purified antibodies from both rabbits gave very weak, uninformative immunostaining patterns.

## 3.4. Modification of antibody purification techniques

To obtain a working UNC-45 antibody for immunostaining I tried various methods of modifying the purification techniques of all sera I had accumulated (from rabbits 7N5, 3E2, 3E5, 4E1, and 4E5).

### *3.4.1. GST strip purification*

Due to the difficulties in isolating the UNC-45(18-76)-only fragment away from GST I decided to try and remove GST antibodies from my sera by strip purification. I bound GST protein to a nitrocellulose filter and incubated all of my purified antibodies with this filter. Any GST antibodies present should bind to the membrane and the supernatant should be enriched for UNC-45(18-76) antibodies, which could then be concentrated. Through Western analysis I showed that GST antibodies were successfully removed from the polyclonal GST::UNC-45(18-76) antibodies (Figure 3.7). However, when tested on immunostaining there was no improvement in the specificity of any of the GST-removed antibodies compared to the antibodies that had GST present. I therefore concluded that GST may not be the main cause of the lack of specificity.

### *3.4.2. Various fixation procedures*

Each fixation method allows different epitopes to be exposed for antibody binding. The Finney Ruvkun fixation procedure that I had been using uses paraformaldehyde, which forms methylene cross-links between proteins. It is possible that the polyclonal antiserum recognizes epitopes that are not available with this kind of fixation. As an alternative, I tried Bouins Fixative (Nonet et al., 1997). This has been reported to work well for antibodies that do not recognize their epitopes in paraformaldehyde fixed *C. elegans* (Nonet et al., 1997). Bouins fixative combines cross-linking of protein through paraformaldehyde and picric acid with denaturing fixation through methanol. I fixed adult *C. elegans* with Bouins fixative and tested all my purified antibodies on these worms. This method improved some of the antibody specificity (especially 3E2) and resulted in patchy but specific staining of the body wall thick filaments. However, the 3E2 antibody that gave the best staining was in too limited supply (~20 μL), for all of my proposed experiments. Subsequent attempts to purify a new 3E2 antibody from the serum were not satisfactory.

### *3.4.3. Cleavage columns*

For my last attempt to obtain a usable UNC-45 antibody I decided to modify the CNBr antigen affinity column. Given my lack of success in purifying the small UNC-45(18-76) fragment, instead of binding intact GST::UNC-45(18-76) protein to the column I added GST::UNC-45(18-76) protein that had been cleaved by the thrombin cleavage kit (Sigma), thereby having the UNC-45 fragment physically separated from GST. GST could be folding in a way that makes the UNC-45 epitopes inaccessible to antibodies. By cleaving the protein and binding both parts separately, any UNC-45 epitopes that were masked by GST in the fusion should now be exposed. I ran 3E2, 4E5, 4E1, and 7N5 serum over this cleavage column and tested the antibodies on immunostaining using both Finney Ruvkun and Bouins fixative. The 4E1 cleaved antibody yielded very good specificity and I saw the expected pattern for UNC-45 upon immunostaining (Figure 3.8). The MHC B region of the thick filaments was clearly stained and the vulval muscles were seen in some worms. I could not detect any pharynx staining but it is an organ that is deep inside the body and may not have been fully accessible to antibodies following permeabilization. I performed immunostaining multiple times on N2 worms to confirm this antibody was working consistently.

### 3.5. Verifying the UNC-45 antibody

I performed a series of tests to determine if my antibody displayed the same pattern as previously reported (Ao and Pilgrim, 2001; Ao, 2001). Western analysis was performed against worm lysate, in which a band was detected at 109 kDa (the predicted size of full length UNC-45) (Figure 3.9). Other bands of smaller size are also seen which may be degradation products of UNC-45. I also performed a competition assay with excess fusion protein (See Figure 3.9). After incubating the antibody with excess protein, the signal on both a Western blot and immunostaining is greatly reduced. I also compared the UNC-45 immunostaining to both MHC A and MHC B (Figure 3.10). My results show that UNC-45 co-localizes with MHC B, but not MHC A, in the body wall muscles of *C. elegans*, consistent with earlier reports (Ao and Pilgrim, 2001). I also examined UNC-45 staining in some mutant strains and again found that staining patterns were consistent with earlier reports. UNC-45 staining was absent in *unc-54(0)* worms, in which there is no MHC B present in the thick filaments (Figure 3.11a). UNC-45 also did not localize to the thick filaments in an *unc-54(0);sup-3* mutant where MHC A is over-expressed and makes up the entire length of the thick filament (Figure 3.11b). Despite the plethora of technical problems, the results in Figures 3.9-3.11 indicate that I was finally able to produce a suitable UNC-45 antiserum for use on subsequent assays.

### 3.6. Immunostaining and UNC-45 localization in worms containing the chimeric myosins

To determine which (if any) region of MHC B is required for UNC-45 localization to the thick filament in body wall muscles I took advantage of strains of *C. elegans* generated by (and kindly provided by) Dr. Pamela Hoppe (Hoppe and Waterston, 1996) (Figure 3.12 and Table 3.1). These strains each have an extrachromosomal array, which contains a DNA construct driving muscle-specific expression of a chimeric body wall myosin. The chimeras contain various regions of MHC A and MHC B fused together to create a full length myosin. The arrays are present in an *unc-54(0)* (MHC B) background so that the chimeric myosin is their only source of MHC B, allowing domain analysis as each chimeric myosin has a different deleted region of MHC B. The arrays are also marked with different markers (GFP reporter, roller mutation, and/or an HA tag on the chimeric myosin) so that the array can be followed independent of the *unc-54* rescue. Using immunofluorescence I examined UNC-45, MHC A and MHC B localization in all the chimeric strains to determine which chimeric myosins allowed for localization of UNC-45 to the thick filament, indicating the region of MHC B that is required for localization of UNC-45.

The control chimera, B:B:B (full length MHC B), showed normal UNC-45, MHC A, and MHC B localization, which is consistent with a rescuing construct (Figure 3.13). Chimeras A:A$_2$/B:B (Figure 3.14) and B:B/A:A (Figure 3.15) also showed a wild type UNC-45 pattern, with UNC-45 localizing to the MHC B region of the thick filament. Chimeras B:A:A, B:A:Δ, B:A:B, and A:A$_1$/B:B all failed to show thick filament localization of UNC-45 (Figures 3.16, 3.17, 3.18, and 3.19 respectively). UNC-45 staining was still present in the cells, but diffuse in the cytoplasm rather than localized to the filaments. MHC B staining was normal in Chimeras A:$A_1$/B:B and A:$A_2$/B:B but absent in Chimeras B:A:A, B:A:Δ, B:A:B, and B:B/A:A due to the absence of the epitope for the MHC B DM 5-8 monoclonal antisera in the chimeric myosins. In all of these chimeras (except B:B:B) the MHC A DM 5-6 antibody displayed an immunostaining pattern that appeared to span the entire thick filament (i.e. both MHC A and MHC B regions); however, it seemed to preferentially bind to the MHC B region in some strains (See Section 3.7). Chimera A:B:B showed UNC-45 mis-localization as well, present in the cytoplasm around the filaments (Figure 3.20). DM 5-6 gave a wild type MHC A localization pattern and there was no detectable MHC B staining indicating the epitope was missing. From the information provided on this chimera it should contain the MHC B epitope so I expected to see a wild type MHC B pattern. I also saw a high proportion of worms that display wild type movement without the dominant marker present on the extrachromosomal array (*rol-6)* indicating they might not be in an *unc-54(0)* background. Due to this discrepancy (and the fact that Dr. Hoppe was also unsure of the genotype of the strain), the strain may have been mislabeled, and we cannot draw any conclusion from the staining pattern of worms.

When all the data from the chimeras were compared, it looks as if it is the rod region of MHC B that is necessary for the correct localization of UNC-45 to the thick filament. This contrasts with the original hypothesis and previous *in vitro* data that suggested the head would be required for the correct localization of UNC-45 (Figure 3.21).

I attempted to stain the worm strains with phalloidin, which stains actin in the thin filaments, to use as an independent marker for UNC-45 localization in the muscle cells. Unfortunately, phalloidin is incompatible with the fixation method necessary to allow staining with UNC-45. I therefore could not use the thin filaments to gain any additional information as to where the UNC-45 is mis-localizing in the body wall muscle cells.

### 3.7. DM 5-6 antibody (MHC A) preferentially localizes to the MHC B region in some of the chimeras

Since some of the chimeric myosins are tagged with HA, I wanted to determine exactly where the chimeric myosins are localizing in the thick filament. By staining the worms containing the HA chimeric myosins with an anti-HA antibody, I found the chimeras localized exclusively to the MHC B region, leaving an unstained central gap where MHC A is found. This indicates only wild-type MHC A composes the central region of the filament and the chimeric myosins are somehow excluded. It is likely that wild-type MHC A is much more efficient at filament initiation than the chimeric myosins so it preferentially is used in that region. The chimeric myosins then take the place of the missing MHC B to form the filament arms. If this is how the filament is formed in these worms than the DM 5-6 antibody would show a pattern in which both regions of the filament were staining due to the presence of the epitope in MHC A and the chimeric myosins. In all the chimeras, except for B:B:B, I do see the expanded pattern of the DM 5-6 antibody. However, it also appeared in some worms that the DM 5-6 antibody was preferentially staining the MHC B region of the thick filament and leaving a weaker staining pattern in the MHC A region. This could be due to the altered conformation of the chimeric myosins, which allows the epitope for DM 5-6 to be preferentially exposed compared to wild-type MHC A. Chimera B:B:B shows a wild-type MHC A pattern because there is no MHC A present in the chimera so the DM 5-6 antibody has no competing binding sites.

### 3.8. Requirements for UNC-45 in the assembly of the chimeric myosins

Although UNC-45 appears to be mis-localized in worms carrying some of the chimeras, these strains still show wild type movement, suggesting that localization of UNC-45 to the thick filament is not necessary for full function of the chimeric myosin. Based on this observation I wanted to determine if any of the chimeras could properly assemble into thick filaments in the absence of UNC-45. In order to do this I needed to get rid of UNC-45 activity in the strains carrying the chimeric myosin. I did this by crossing the *unc-45(r450)* mutation into the chimeric worms and selecting for double *unc-54(0);unc-45(r450)* mutants. I then tested for the ability of the chimeric myosins to assemble properly into the thick filaments by growing the double mutants at the restrictive temperature for the *unc-45(r450)* allele (20°C) and scoring for the ability to move. I expected that the chimeric myosins that contain the MHC B head would not be able to properly assemble without UNC-45 due to their requirement for the UNC-45 chaperone activities. Since MHC A does not seem to require UNC-45 to help it assemble (Ao and Pilgrim, 2000) the constructs that contain the MHC A head should be able to assemble and these worms were expected to show a nearly wild-type movement in an *unc-45* mutant background.

To determine whether I had isolated an *unc-54(0);unc-45(ts);*transgenic array strain, I needed to score for the loss of the extrachromosomal array, which should result in paralyzed worms. I was not able to do this with Chimeras B:B:B and B:A:A because they showed nearly 100% transmission of the transgenic array. I am not sure whether these arrays are integrated or whether they just very stable in meiosis. I would assume that Chimera B:B:B would result in paralyzed worms in an *unc-54(0);unc-45(r450)* mutant grown at the restrictive temperature. Since this chimera contains the full length MHC B it would be forming the same kind of filament that forms in an *unc-45(r450)* single mutant (that has wildtype MHC B). These worms are paralyzed indicating that full length MHC B does need UNC-45 in order to assembly properly at the thick filament. I could also not obtain a double mutant with chimera B:B:A due to the loss of this strain. Crosses to verify if I obtained a double mutant indicated I only had single mutants with worms carrying Chimeras B:A:B and A:$A_1$B:B (Figures 2.2 and 2.3, Table 3.2). After verifying mutants carrying chimeras B:A:Δ and A:$A_2$/B:B I concluded that I had obtained double mutants for these two strains (See Figures 2.2 and 2.3, Table 3.2). The double mutant carrying the chimera B:A:Δ was paralyzed at the restrictive temperature indicating that UNC-45 is essential for thick filament formation when this chimeric myosin substitutes for MHC B in the thick filament. The double mutant carrying chimera A:$A_2$/B:B was capable of movement at the restrictive temperature. This indicates that when this chimera substitutes for MHC B in the thick filament UNC-45 function is not necessary to assembly a functional filament (Figure 3.22).

The major difference between these two chimeras is the composition of the head. In the double mutant containing chimera A:$A_2$/B:B the head is composed of MHC A whereas the double mutant containing chimera B:A:Δ is composed of MHC B. These data suggest that the MHC B head is dependant on UNC-45 to help it fold and form a functional filament. The MHC A head does not seem to have the same requirement and is able to fold and form a functional filament without the help of UNC-45. B:A:Δ is also missing the tail region, suggesting that this region could also be necessary to assemble a functional filament.

### 3.9. Domain analysis of UNC-45

As discussed earlier, UNC-45 protein includes the TPR, Central, and UCS domains (Venolia *et al.*, 1999). In order to assess the functions of these three domains I created deletion constructs containing domains singly or in combinations. I attempted to create TPRonly, TPR/Central, and UCSonly deletion constructs with the UNC-45 promoter and an in frame fusion to GFP at the C terminus (Figure 3.23). Due to technical difficulties, I was unable to produce the Central/UCS domain construct (see Section 2.12). First, I injected the remaining constructs into wild-type (N2) worms to determine which region of UNC-45 is required to bind the MHC B region of the thick filament. Full length UNC-45, when injected into *unc-45(ts)* worms, is able to rescue the mutant phenotype by improving both mobility and muscle structure (Ao, 2001). I wanted to test whether any of the deletion constructs could rescue the mutant phenotype. I first injected the constructs into wild-type N2 worms to see localization pattern. I next injected the constructs into *unc-45(r450)* worms, selecting GFP positive progeny, and examining them for improved mobility. I did this by injecting worms grown at the permissive temperature (16°C) and allowing them to transmit to the first generation at 16°C. I then transferred the next generation to the restrictive temperature to see if the deletion constructs allowed for rescue.

The TPRonly and UCSonly fragments of UNC-45 were the only transgenic lines I was able to produce. Both showed mosaic expression in the N2 worms, with each worm displaying a slightly different GFP expression pattern. I could find no discernable differences between the two GFP expressing constructs. The expression also decreases as the animal ages to the point it is almost undetectable in some worms. I also found high embryonic lethality as well as L1 arrest. These embryos and L1 arrested larvae are similar in that they have high expression of GFP (Figure 2.24), compared to the surviving embryos that had low levels. This indicates that high levels of the TPRonly and the UCSonly fragments could be lethal. I could also not obtain any stable lines of *unc-45(r450)* worms injected with the constructs so I could not transfer any to the restrictive temperature and score for rescue.

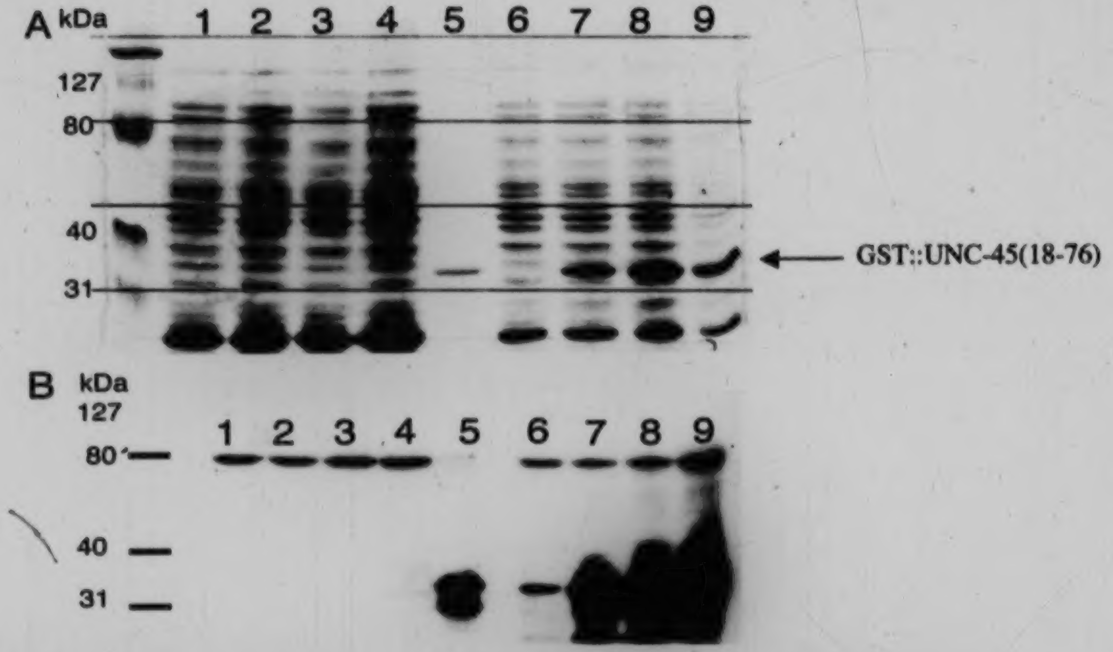


**Figure 3.1: Western analysis of 7N5 antisera against GST::UNC-45(18-76).** A. Coomassie staining shows specific induction of GST::UNC-45(18-76) in BL-21 cells. Lanes 1-4 are BL-21 cells only. Lane 5 is GST::UNC-45(18-76) after purification. Lanes 6-9 are BL-21 cells induced for GST::UNC-45(18-76) expression at various time points (1-4 hours after induction respectively). B. Western analysis of A using 7N5 antisera. Note there is no reaction to the 7N5 antisera in BL-21 cells only(Lanes 1-4). After the induction of GST::UNC-45(18-76), the 7N5 antisera shows a very strong reaction as induction time progresses. 7N5 antisera and the 2° was used at a dilution of 1/20000. The 7N5 antisera recognizes purified GST::UNC-45(18-76) protein used to induce the response of the rabbits

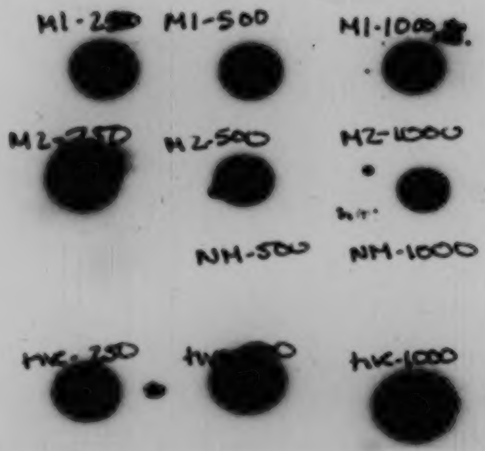

M1-250
M1-500
M1-1000
M2-250
M2-500
M2-1000
NM-500
NM-1000
+ve-250
+ve-1000

**Figure 3.2: Dot blots of monoclonal mouse serum.** Dilution of serum is indicated above each dot. Secondary anti-mouse and rabbit (positive control) was used at 1/10000. A normal mouse (NM) showed no reaction to GST::UNC-45(18-76) while the polyclonal 7N5 control showed a strong reaction. Mice boosted for monoclonal antibody production mounted an immune response to GST::UNC-45(18-76). M1 was chosen for the fusion reaction to create a monoclonal antibody.

201
127
80
40
31

**Figure 3.3 : Western analysis of the monoclonal positive colonies.** Two positives were identified for a response against GST::UNC-45(18-76). The two positive colonies were too sick to perform single cell cloning and eventually all the cells died before a monoclonal antibody could be made. Supernatants were used at a dilution of 1/10 and 2° anti-mouse was used at 1/10000.

**Figure 3.4: Immunostaining with polyclonal antibodies generated from mouse serum.** Antibodies showed a variety of staining patterns. A and B are sera from mouse M1. (A) shows vulval muscle staining and (B) shows body wall filament staining. The stained filaments do not appear to correspond to the MHC B region as previously described (refer to Figure 3.5 for comparison)

**Figure 3.5: Immunostaining with purified 3E2 antibody.** Antibody staining localized to the MHC B region of the thick filaments (A) and the vulval muscles (B) of adult *C. elegans*. This is the same pattern seen with the previous UNC-45 antibody made in our lab (Ao, 2001). 3E2 antisera was used at a dilution of 1/250 and Alexa 488 anti-rabbit was used at 1/1500.

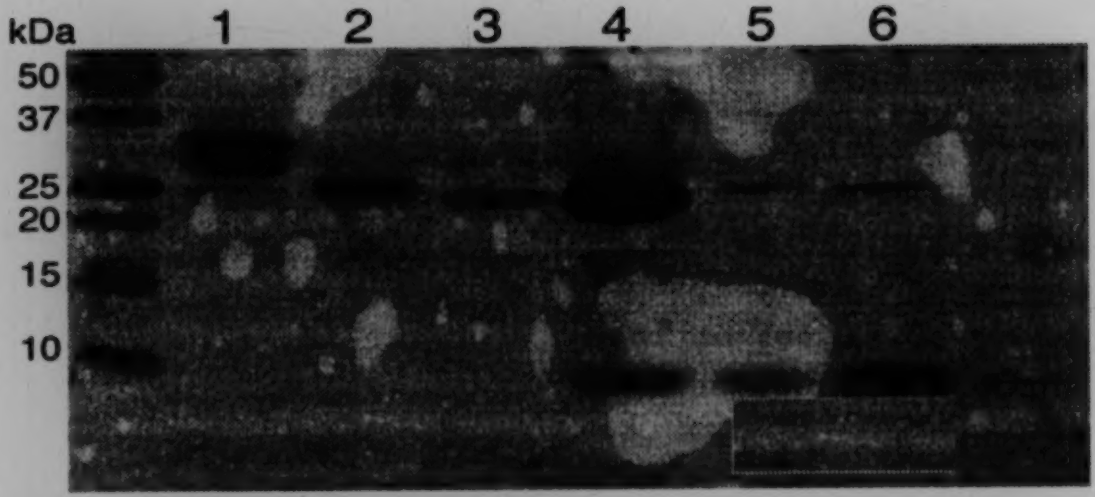


**Figure 3.6: Thrombin cleavage performed on GST::UNC-45(18-76).** Cleavage released the UNC-45(18-76)only fragment. Lane 1 is GST::UNC-45(18-76), Lane 2 is GST protein, Lane 3 is GST protein isolated after cleavage, Lane 4 is cleavage reaction, Lanes 5 and 6 are UNC-45(18-76) only (5 uL and 10 uL respectively). The arrow indicates the 6 kDa UNC-45(18-76) band. This fragment was unstable and degraded very quickly so could not be used to inject rabbits or make an UNC-45(18-76) specific column.

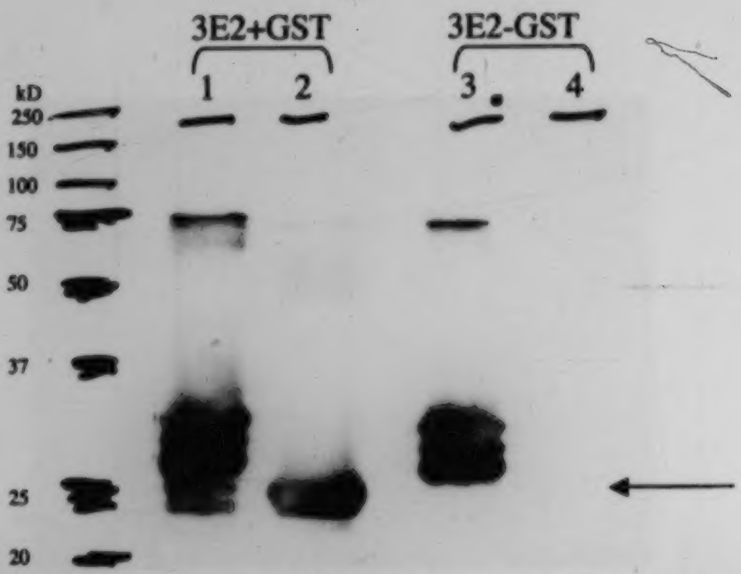


**Figure 3.7: Removal of GST antibodies present in 3E2 antiserum.** GST antibodies present in the 3E2 polyclonal UNC-45 antibody were successfully removed. Lanes 1 and 3 are GST::UNC-45(18-76) protein and lanes 2 and 4 are GST only protein. Lanes 1 and 2 were incubated with 3E2 antibody+GST and lanes 3 and 4 were incubated with 3E2 antibody after GST was removed. Both were diluted to 1/5000 and 2° was used at 1/10000. MW of GST is 26 kDa and MW of GST::UNC-45(18-76) is 33 kDa. Note that there is no reaction to the GST only protein when incubated with 3E2-GST.

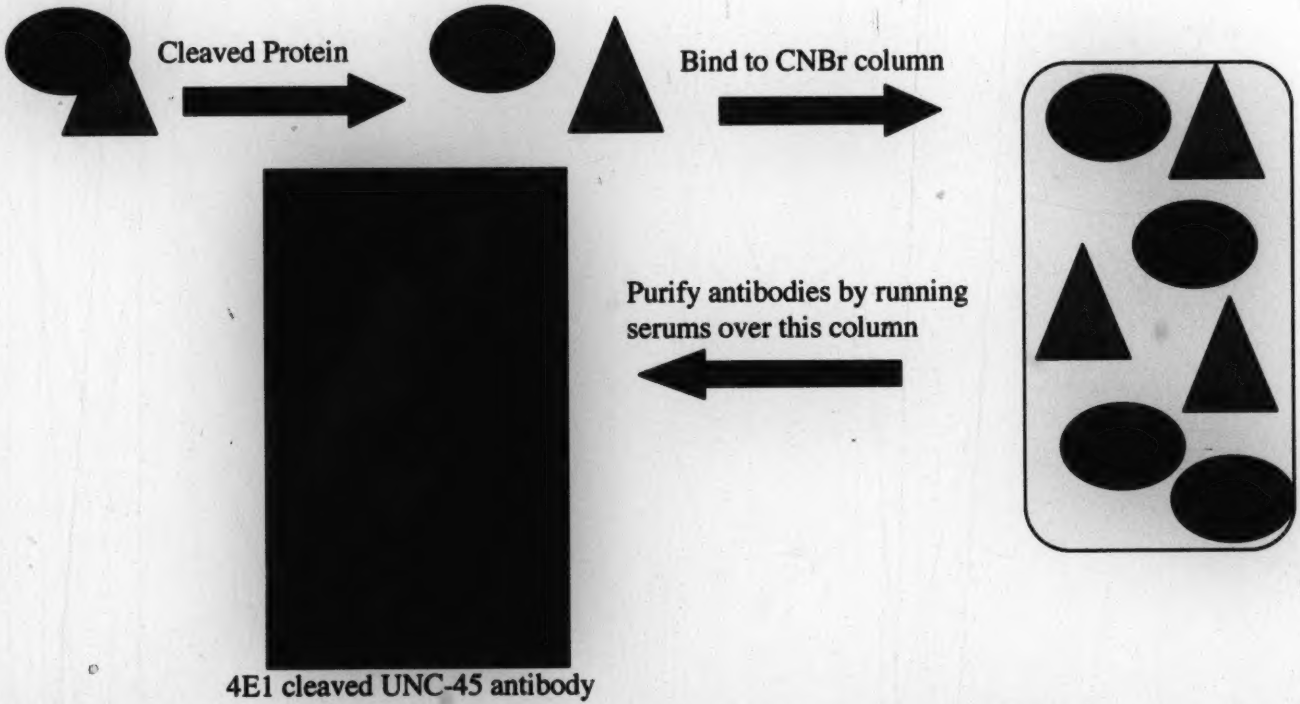

Cleaved Protein
Bind to CNBr column
Purify antibodies by running serums over this column
4E1 cleaved UNC-45 antibody

**Figure 3.8: Method used to obtain a reliable UNC-45 antibody**. GST::UNC-45(18-76) was cleaved by thrombin to release the UNC-45 fragment from GST. See Figure 3. 6 (Lane 4) for protein extract used to bind the column. Both fragments were bound to a CNBr column and 4E1 serum was run over the column and purified. The resulting antibody gave the expected thick filament staining pattern (MHC B region). 4E1cleaved antibody was used at a dilution of 1/250 and 2° Alexa 488 anti-rabbit was used at 1/1500.

1
2
3
4
250
150
100
75
50
37
A
B
C

**Figure 3.9: Western analysis and competition assay of 4E1cleaved antiserum.** The 4E1 cleaved antibody recognizes native UNC-45 protein and is outcompeted with the addition of GST::UNC-45(18-76). (A) shows a Western Blot performed on worm lysate using the 4E1cleaved antiserum. Lanes 1 and 2 have no added competitor and show the expected 109 kDa band. Antisera in lanes 3 and 4 were incubated with an excess of GST::UNC-45(18-76) and the 109 kDa band is reduced or eliminated. Antisera in all lanes were used at a dilution of 1/5000 and 2° antibody was used at a dilution of 1/20000. (B) is an adult worm stained with 4E1cleaved at a dilution of 1/5000. (C) is 4E1 staining (1/5000) after incubation of the antisera with excess GST::UNC-45(18-76) overnight. Note that the thick filament pattern is absent in (C), indicating the 4E1cleaved signal is specific to GST::UNC-45(18-76).

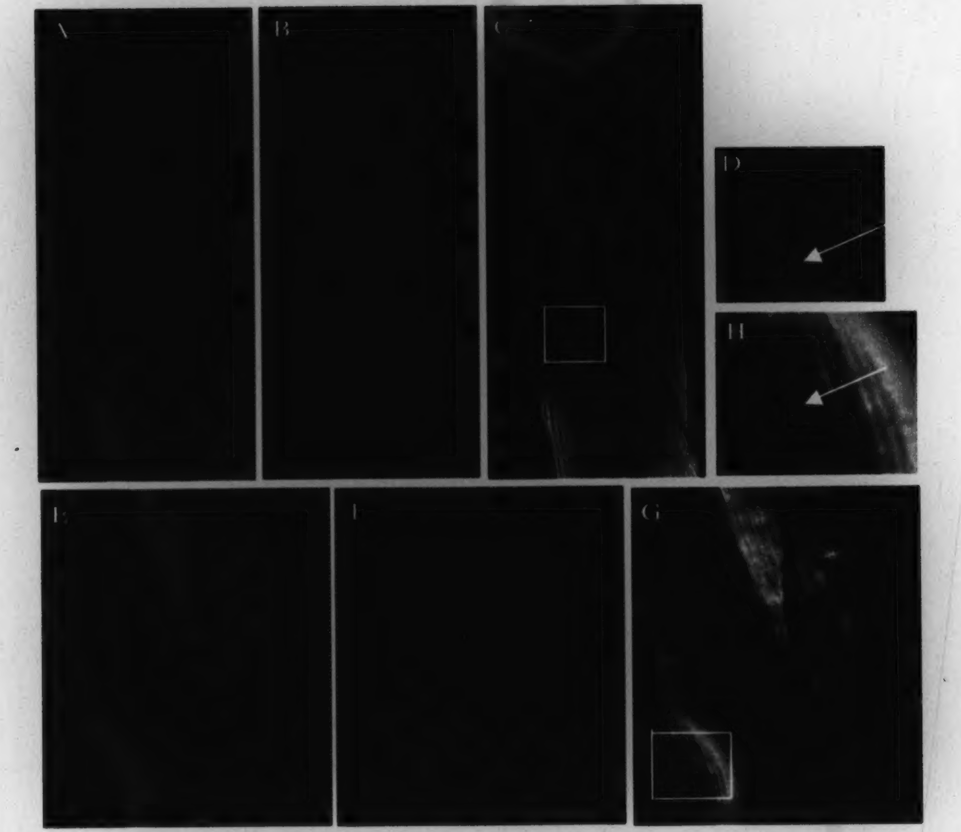


**Figure 3.10: Immunostaining on N2 worms with 4E1cleaved antibody.** UNC-45 co-localized with MHC B in the thick filaments but not with MHC A. A, B, and C are the same field of the body wall muscle labeled with 4E1cleaved (UNC-45), DM 5-6 (MHC A), or double labeled with 4E1cleaved and DM 5-6 respectively. D is an enlargement of the white box in C. E, F, and G are the same field of the body wall muscle labeled with 4E1cleaved (UNC-45), DM 5-8 (MHC B), or double labeled with 4E1cleaved and DM 5-8 respectively. G is an enlargement of the white box in G. Arrows in D and H indicate the central MHC A region where there is is no localization of UNC-45.

A
UNC-45
B
MHC A
C
Merge
D
MHC B

**Figure 3.11a: Immunostaining of 4E1cleaved UNC-45 antibody in *unc-54(0)* worms.** UNC-45 is not localized to the thick filaments when MHC B is absent. 4E1cleaved, DM 5-6 (MHC A), and Dm 5-8 (MHC B) were all used at a dilution of 1/250. 2° were all used at a dilution of 1/1500.

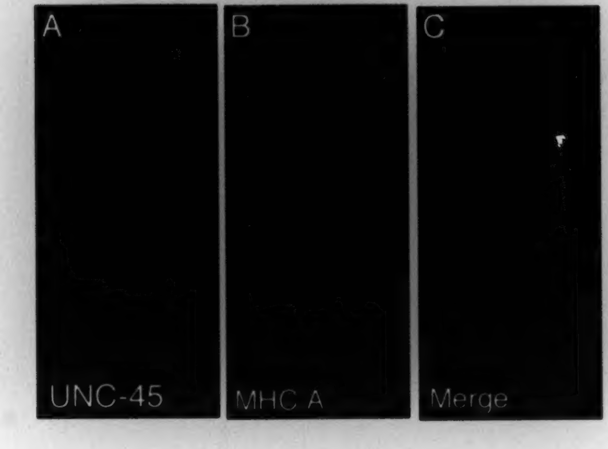
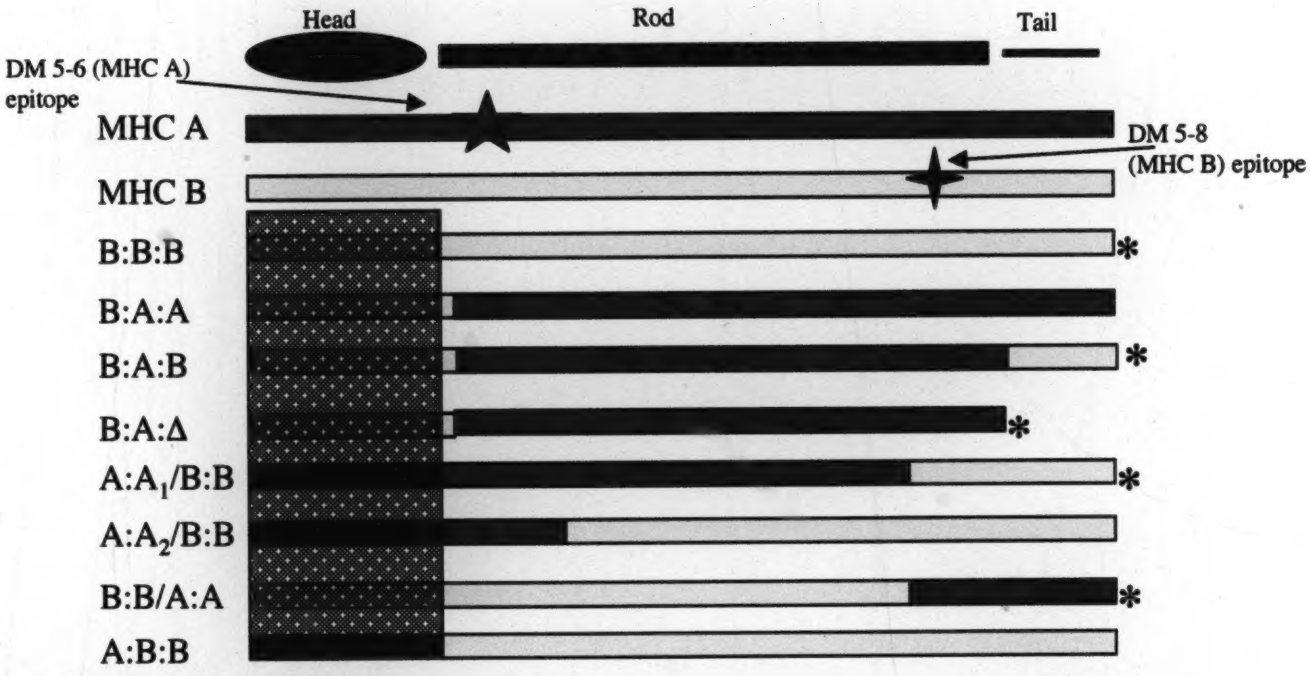


**Figure 3.11b: Immunostaining of 4E1cleaved UNC-45 antibody in *unc-54(0); sup(3)* worms.** UNC-45 is not localized to the thick filaments in worms in which MHC B is absent but thick filament structure is improved through over-expression of MHC A. 4E1cleaved and MHC A antisera were used at a dilution of 1/250. Alexa 2°'s were used at 1/1500.


Head
Rod
Tail
DM 5-6 (MHC A) epitope
DM 5-8 (MHC B) epitope
MHC A
MHC B
B:B:B
B:A:A
B:A:B
B:A:Δ
A:A1/B:B
A:A2/B:B
B:B/A:A
A:B:B

* Chimeras are HA tagged

**Figure 3.12: Diagram of chimeric myosins used in UNC-45 localization studies.** Chimeras are expressed from extrachromosomal arrays in an *unc-54(0)* background (Hoppe and Waterston, 1996). X:X:X indicates whether the head, rod or tail respectively is made up of either MHC A (A) or MHC B (B) sequence (names were changed from Hoppe and Waterston, (1996) for clarity). See Table 3.1 for genotypes of strains carrying chimeric molecules. Immunostaining with 4E1cleaved was performed on worms expressing these chimeras to determine which filaments UNC-45 can localize. The shading indicates the predicted region of UNC-45 localization based on *in vitro* data from Barral et al., (2002).

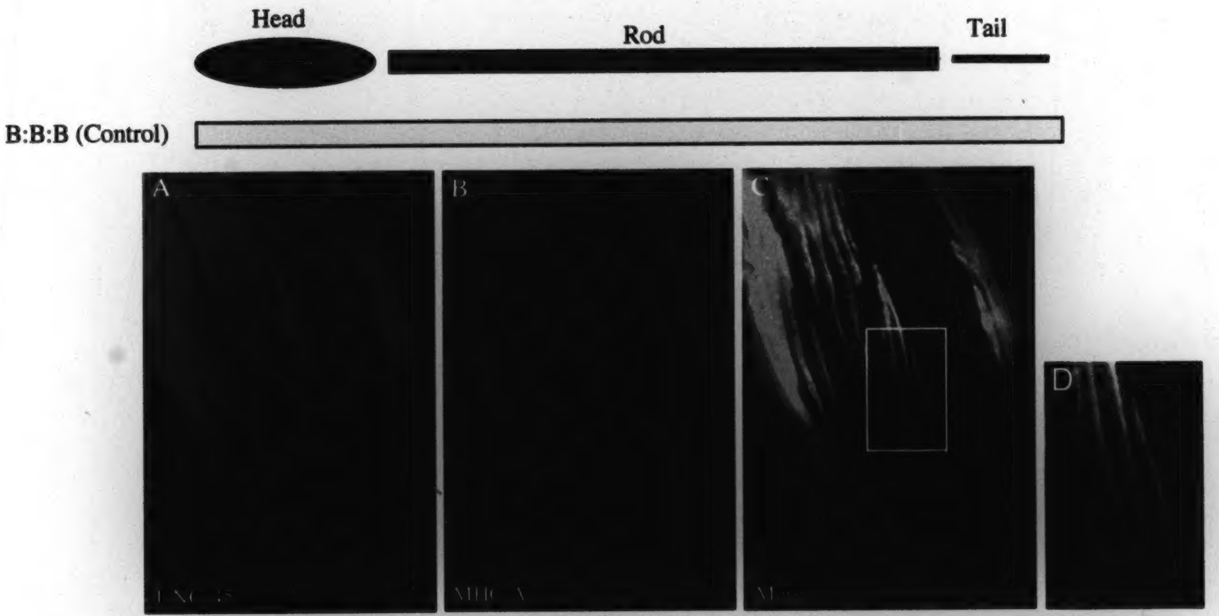


**Figure 3.13: Immuno-localization of UNC-45 in Chimera B:B:B.** UNC-45 is properly localized to the MHC B region of the thick filament with the control construct (full length MHC B). (A) and (B) are the same field of the worm stained with 4E1cleaved and DM 5-6 respectively. (C) is the merge of A and B and (D) is an enlargement of the white box in C to show the central MHC A region (red) and UNC-45 localizing to the MHC B region (green). For figures 3.13 to 3.20 4E1cleaved, DM 5-6, and DM 5-8 were used a dilution of 1/250. Alexa 2°s were used at a dilution of 1/1500.

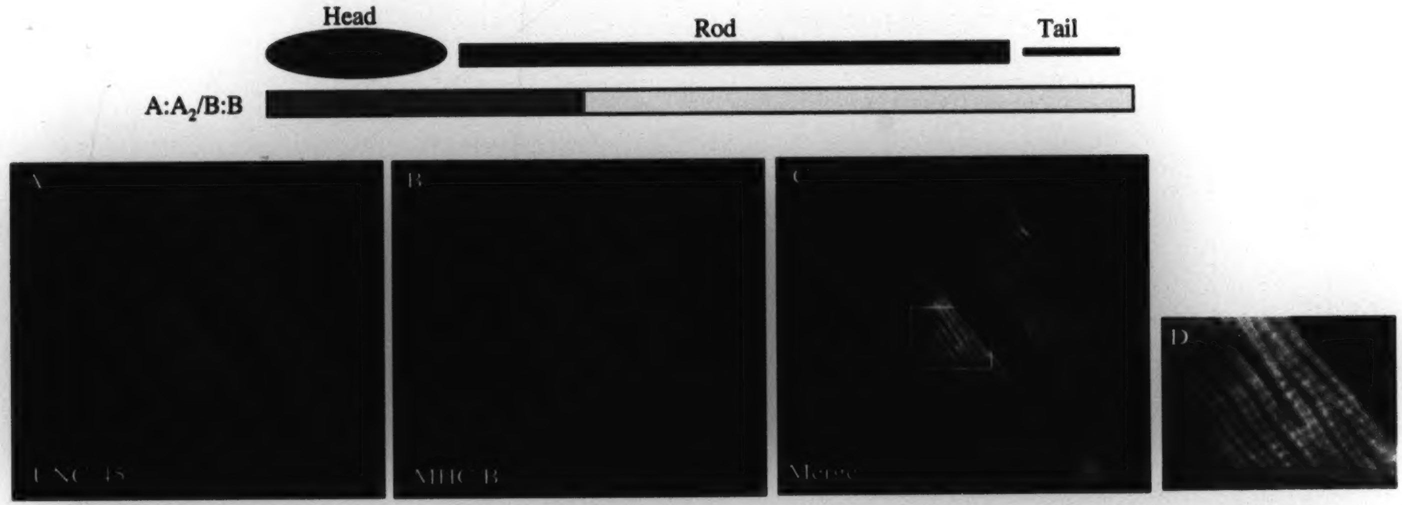


**Figure 3.14: Immuno-localization of UNC-45 in Chimera A:A$_2$/B:B.** UNC-45 is properly localized to the MHC B region of the thick filament when their body wall myosins contain the MHC A head and first part of the rod. The remainder of the rod and the tail are composed of MHC B. (A) and (B) are the same field of the worm stained with 4E1cleaved and DM 5-8 respectively. (C) is the merge of A and B and (D) is an enlargement of the white box in C to show the co-localization of UNC-45 and MHC B to the polar regions of the filament, leaving an unstained central gap. Note: MHC B can be visualized in this strain due to the presence of the DM 5-8 epitope (See Figure 3.12)

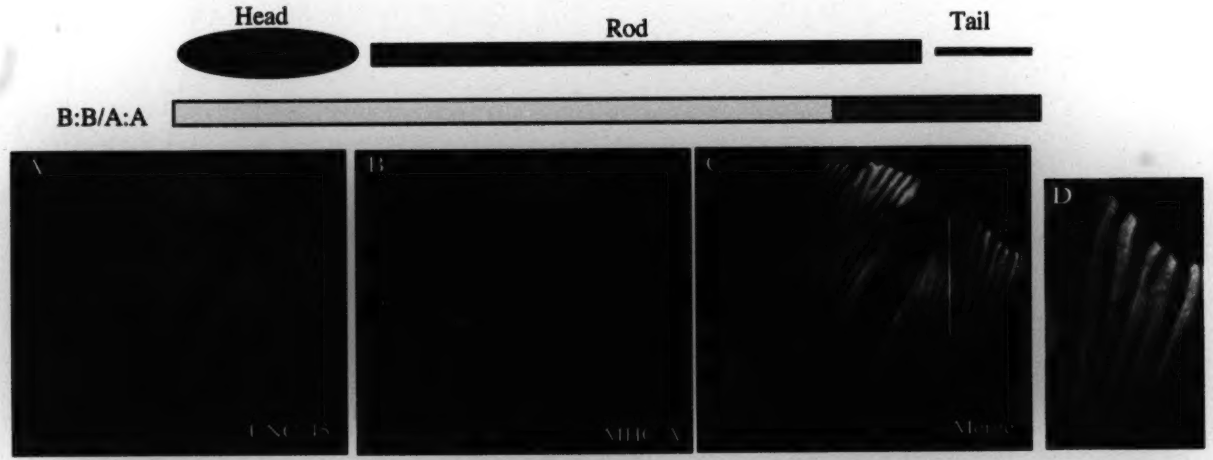


**Figure 3.15: Immuno-localization of UNC-45 in Chimera B:B/A:A.** UNC-45 is properly localized to the MHC B region of the thick filament when their body wall myosins contain the MHC B head and most of the MHC B rod with the remainder of the rod and the tail composed of MHC A. (A) and (B) are the same field of the worm stained with 4E1cleaved and DM 5-6 respectively. (C) is the merge of A and B and (D) is an enlargement of the white box in C to show the localization of UNC-45 to the polar regions of the filament (MHC B), leaving an unstained central gap.

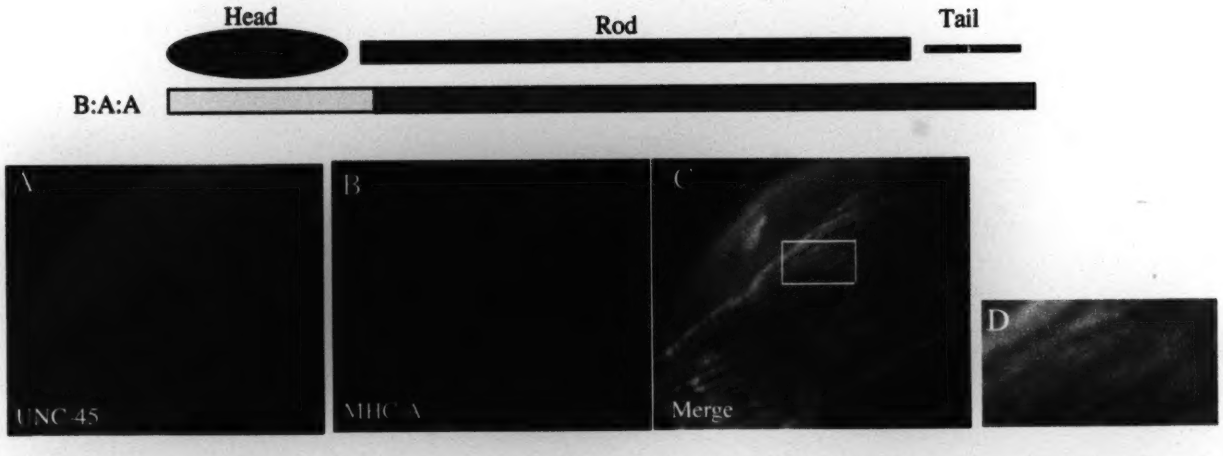


**Figure 3.16: Immuno-localization of UNC-45 in Chimera B:A:A.** UNC-45 is mis-localized in worms when their body wall myosins contain the MHC B head and MHC A rod and tail. (A) and (B) are the same fields of body wall muscles stained with 4E1cleaved and DM 5-6 respectively. (C) is a merge of the two images. (D) is an enlargement of the white box in C showing diffuse UNC-45 staining in the cytoplasm while MHC A is incorporated into the thick filament. Note: MHC B staining could not be observed due to absence of the DM 5-8 epitope.

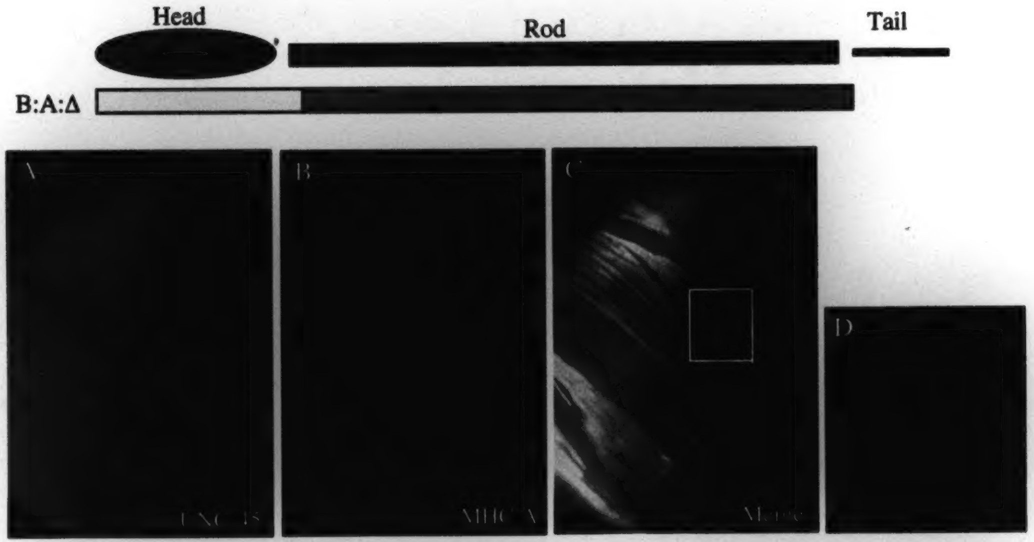


**Figure 3.17: Immuno-localization of UNC-45 in Chimera B:A:Δ.** UNC-45 is mis-localized in worms when their body wall myosins contain the MHC B head, MHC A rod, and no tail. (A) and (B) are the same fields of body wall muscles stained with 4E1cleaved and DM 5-6 respectively. (C) is a merge of the two images. (D) is an enlargement of the white box in C showing diffuse UNC-45 staining in the cytoplasm while MHC A is incorporated into the thick filament. Note: MHC B staining could not be observed due to absence of the DM 5-8 epitope.

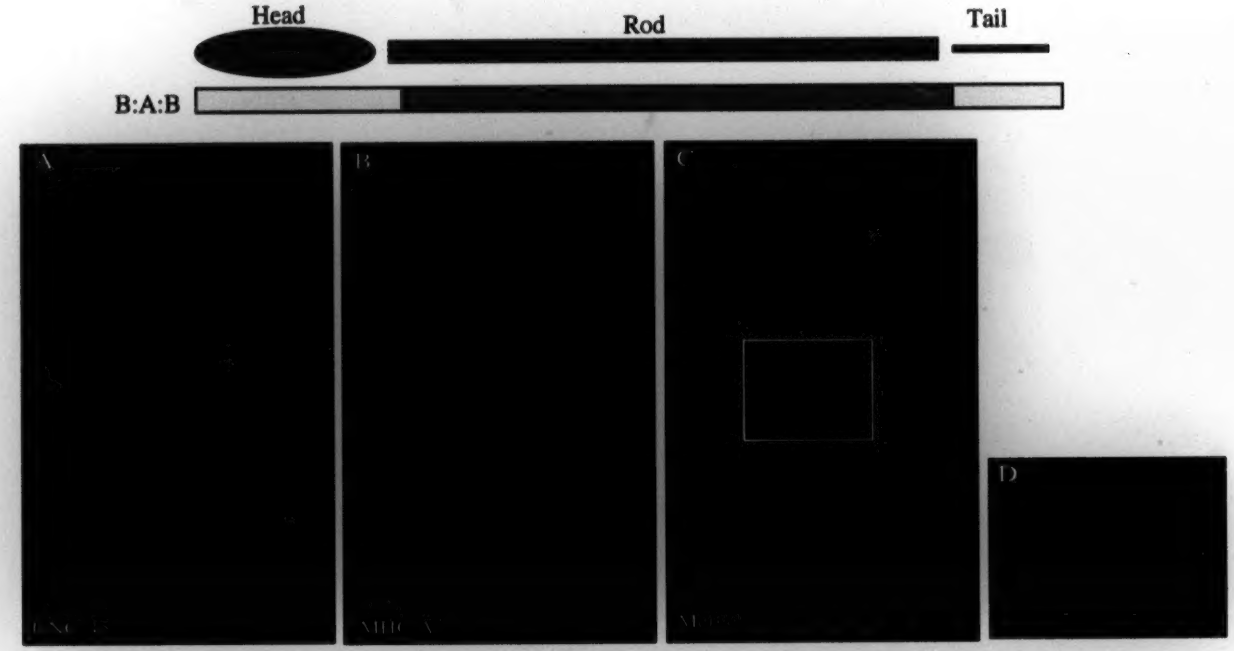


**Figure 3.18: Immuno-localization of UNC-45 in Chimera B:A:B.** UNC-45 is mis-localized in worms when their body wall myosins contain the MHC B head, MHC A rod, and MHC B tail. (A) and (B) are the same fields of body wall muscles stained with 4E1cleaved and DM 5-6 respectively. (C) is a merge of the two images. (D) is an enlargement of the white box in C showing diffuse UNC-45 staining in the cytoplasm while MHC A is incorporated into the thick filament. Note: MHC B staining could not be observed due to absence of the DM 5-8 epitope.

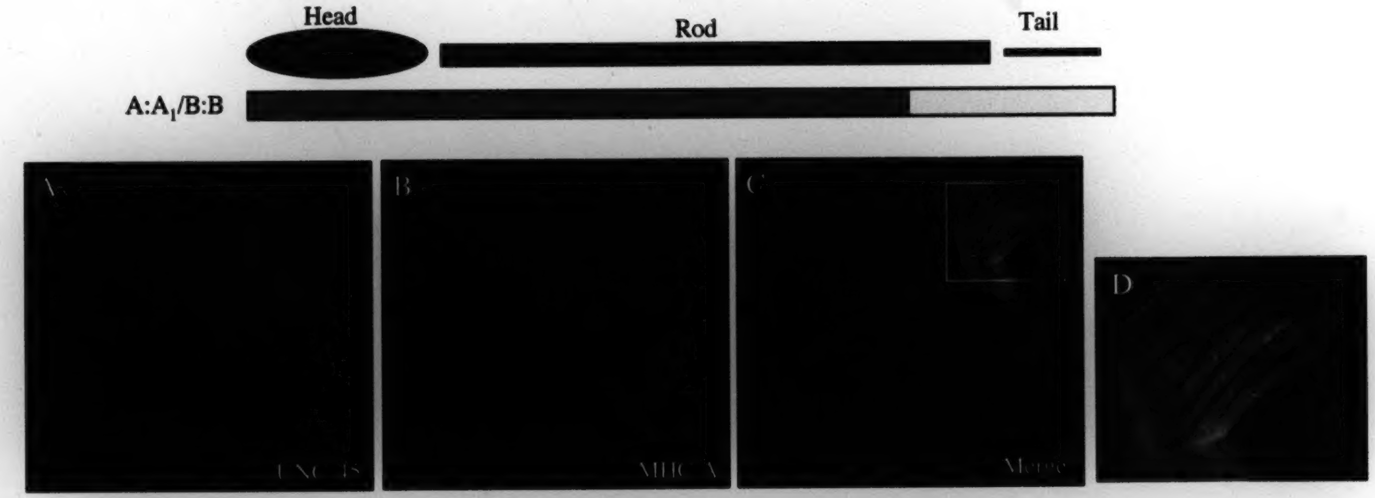


**Figure 3.19: Immuno-localization of UNC-45 in Chimera $A:A_1/B:B$.** UNC-45 is mis-localized in worms when their body wall myosins contain the MHC A head and most of the rod. The remainder of the rod and the tail is composed of MHC B. (A) and (B) are the same fields of body wall muscles stained with 4E1cleaved and DM 5-6 respectively. (C) is a merge of the two images. (D) is an enlargement of the white box in C showing diffuse UNC-45 staining in the cytoplasm while MHC A is incorporated into the thick filament. Note: MHC B staining could not be observed due to absence of the DM 5-8 epitope.

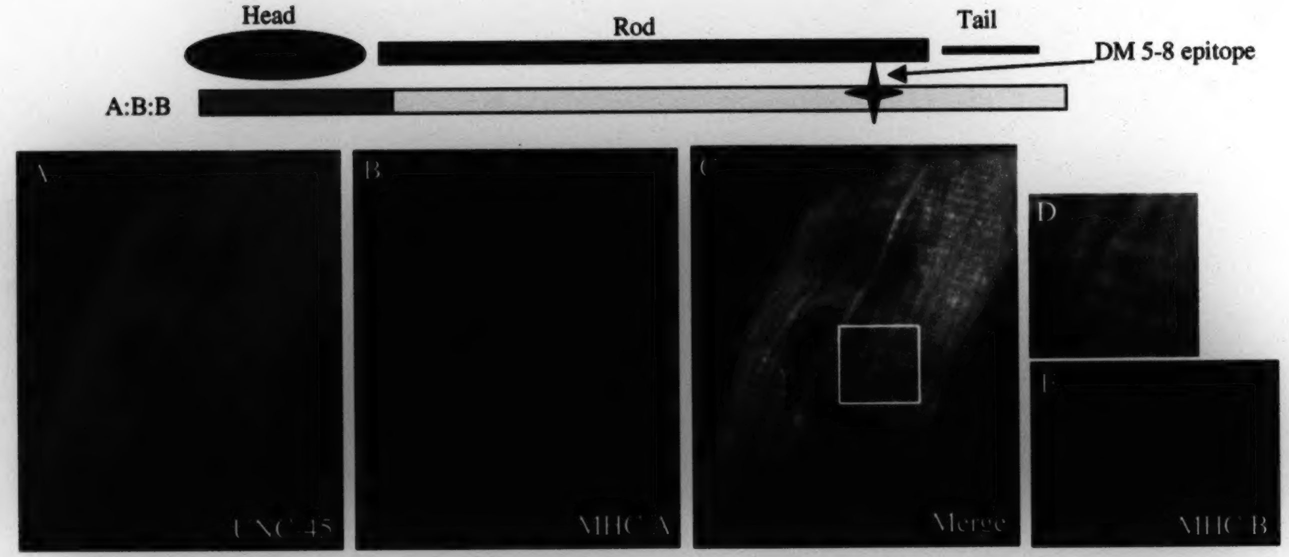
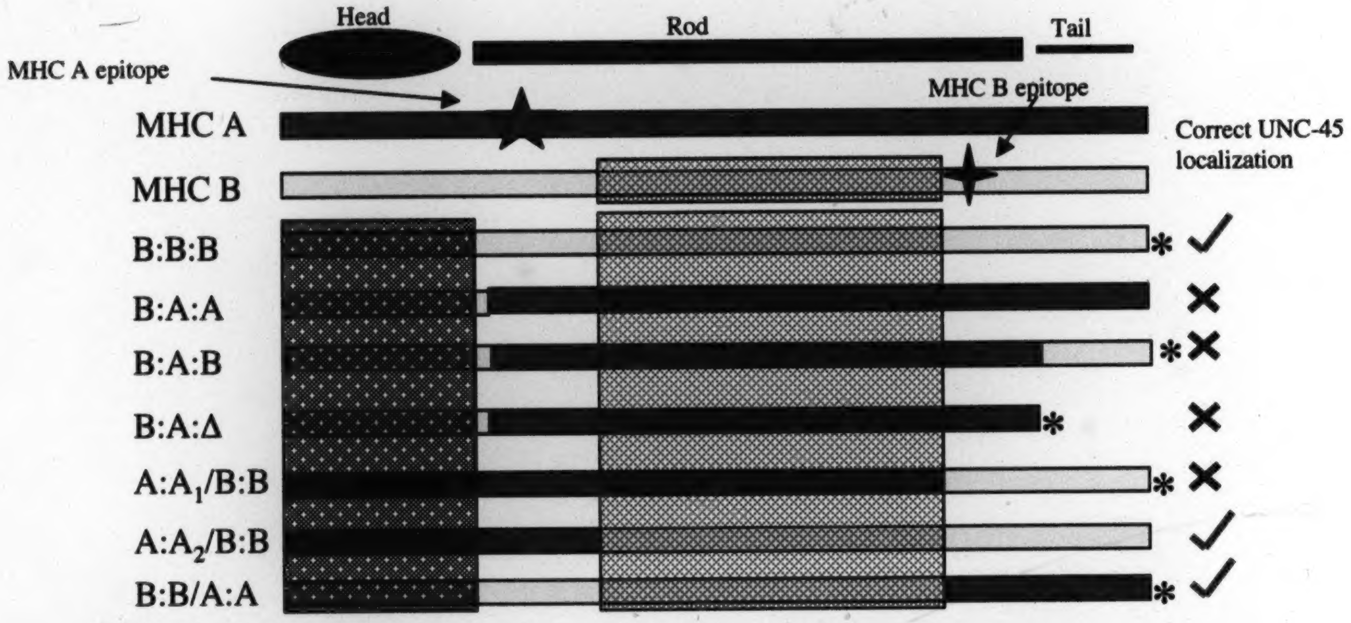


**Figure 3.20: Immuno-localization of UNC-45 in Chimera A:B:B.** UNC-45 is mis-localized in worms when their body wall myosins contain the MHC A head with the rest of the filament composed of MHC B. (A) and (B) are the same fields of body wall muscles stained with 4E1cleaved and DM 5-6 respectively. (C) is a merge of the two images. (D) is an enlargement of the white box in C showing diffuse UNC-45 staining in the cytoplasm while MHC A is incorporated into the thick filament. Note: I am not sure if these worms actually contain the A:B:B chimera. The region of the rod that contains the DM 5-8 epitope is present however there is no staining observed with DM 5-8 (E).


Head
Rod
Tail
MHC A epitope
MHC B epitope
MHC A
MHC B
Correct UNC-45 localization
B:B:B
B:A:A
B:A:B
B:A:Δ
A:A1/B:B
A:A2/B:B
B:B/A:A
*
✓
✗

* Chimeras are HA tagged

**Figure 3.21: Summary of UNC-45 localization studies in worms containing the chimeric myosins** (in an *unc-54(0)* background). UNC-45 is localized correctly to the thick filaments in the worms that contain the region of the rod highlighted in green. The MHC B head did not appear to be required for UNC-45 localization as previously predicted (highlighted in red).

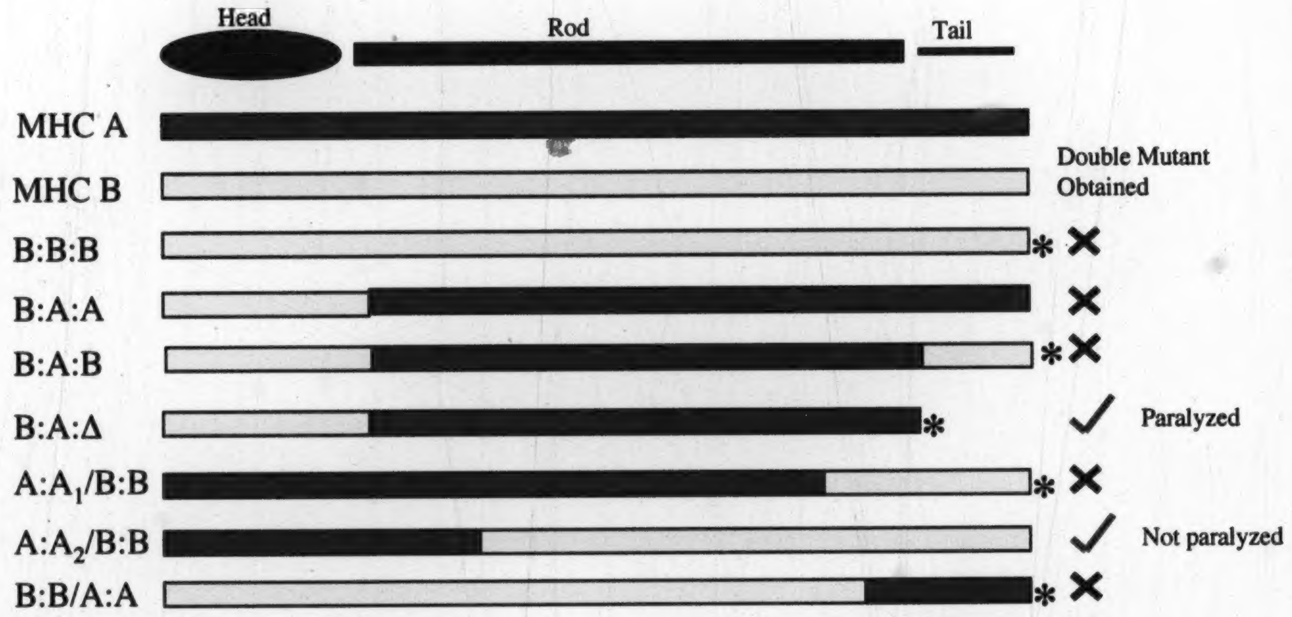

Head
Rod
Tail
MHC A
MHC B
Double Mutant Obtained
B:B:B
*
✗
B:A:A
✗
B:A:B
*
✗
B:A:Δ
*
✓
Paralyzed
A:A$_1$/B:B
*
✗
A:A$_2$/B:B
✓
Not paralyzed
B:B/A:A
*
✗

**Figure 3.22: Summary of *unc-45(r450);unc-54(0)+extrachromosomal array* double mutants**. Only B:A:Δ and A:A$_2$B:B double mutants were constructed. A double mutant with the B:A:Δ chimera resulted in paralyzed worms while the double mutant carrying the chimera A:A$_2$B:B were able to move well in the absence of UNC-45.

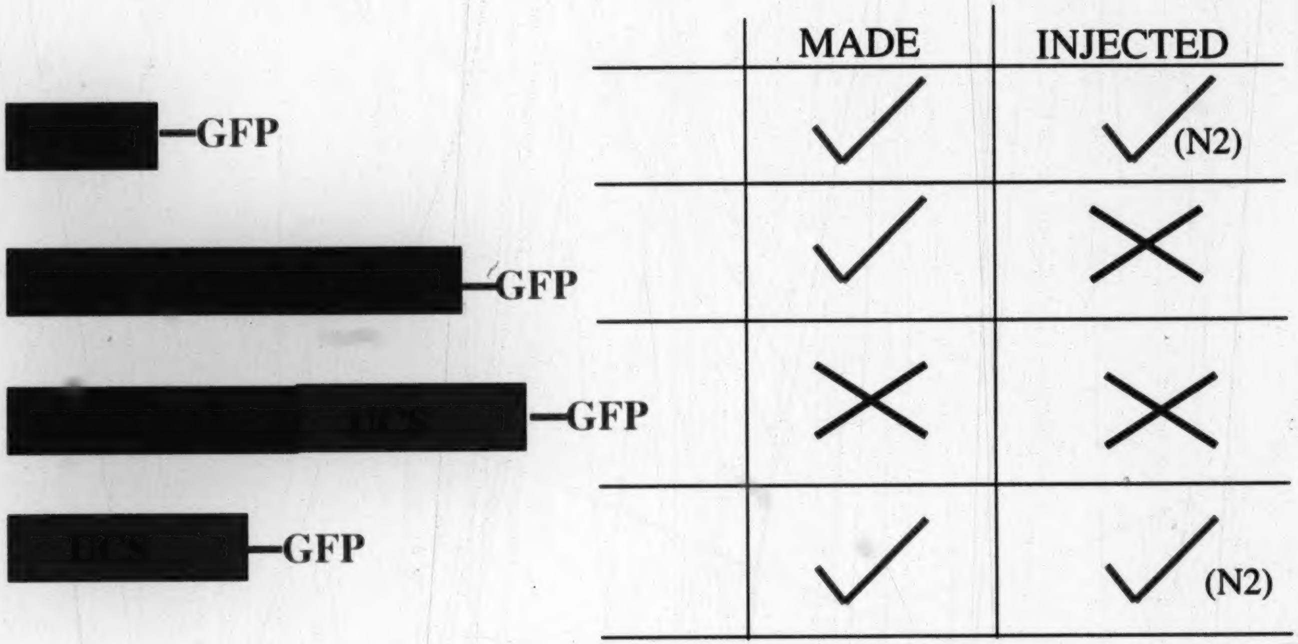

GFP
GFP
UCS
GFP
UCS
GFP
MADE
INJECTED
(N2)
(N2)

**Figure 3.23: Progress made on UNC-45 deletions constructs.** All were made except the Central/UCS. Successful lines were isolated in N2 worms for the TPRonly and UCSonly constructs. GFP expression was mosaic and a clear expression pattern could not be obtained. Lethality was seen in L1 larvae with high GFP expression.

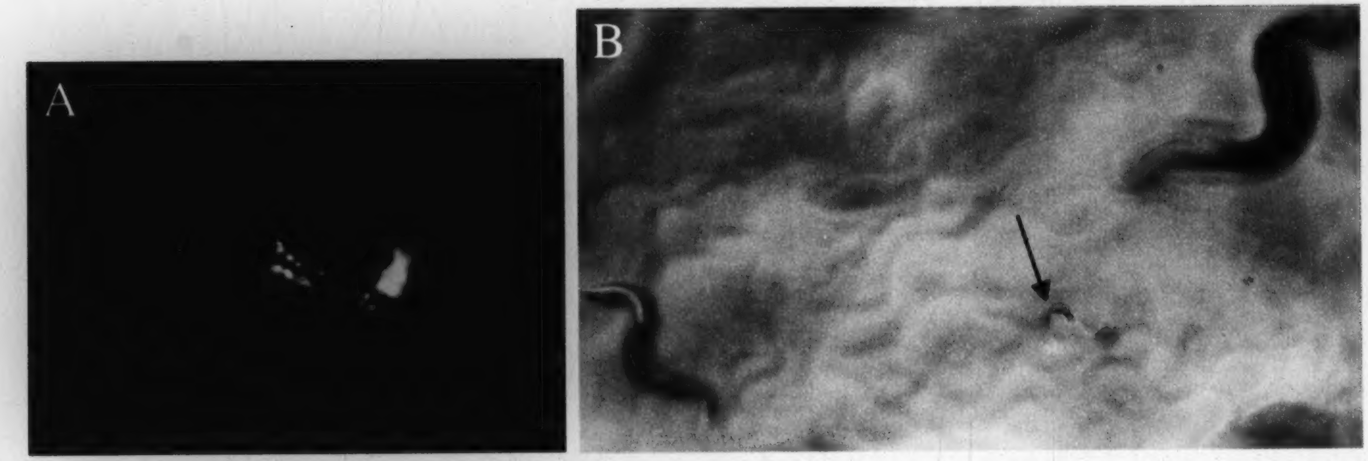


**Figure 3.24: GFP expression in N2 worm injected with UCS only construct**. (A) shows GFP expression and (B) shows the same worm on DIC (indicated by the arrow). The L1 larvae worm did not develop past this stage.

| Chimera name used in this thesis head:rod:tail | Name used in Hoppe and Waterston (1996) | Strain Name | Genotype | Dominant Marker on the Extrachromosomal Array |
|---|---|---|---|---|
| B:B:B | Control | RW3775 | *unc-54(e190);* stEx61 | myo-2::GFP |
| B:A:A | Chimera 3 | RW3768 | *unc-54(e190)*; stEx55 | rol-6::GFP |
| B:A:B | Chimera 4 | RW3811 | *unc-54(e190)*; stEx118 | rol-6::GFP |
| B:A:Δ | Chimera 6 | RW3886 | *unc-54(e190)*; stEx153 | rol-6::GFP |
| A:$A_1$/B:B* | Chimera 7 | RW3779 | *unc-54(e190)*; stEx62 | myo-2::GFP |
| A:$A_2$/B:B* | Chimera 2 | RW3653 | *unc-54(e190)*; stEx85 | rol-6::GFP |
| B:B/A:A* | Chimera 11 | RW3809 | *unc-54(e190)*; stEx116 | rol-6::GFP |
| A:B:B | Chimera 1 | BA lev 2 1e | *unc-54(e190)*; *lev-11(x12)* | rol-6 |

* $A_1$/B = MHC A rod residues S795 - Q1611 (remainder of rod is MHC B sequence)

$A_2$/B = MHC A rod residues S795 - I1079 (remainder of rod is MHC B sequence)

B/A = MHC A rod residues Q1611 - M1933 (remainder of rod is MHC B sequence)

**Table 3.1: Names of chimeric myosin strains used in this thesis.** This table indicates what the original chimeric strains were called and their genotypes as well as what I re-named them for this thesis.

| Chimera Name | Trial # | # of UNC | # of Wildtype | Double Mutant Percentage (%) | Average (%) |
|---|---|---|---|---|---|
| A:A2/B:B #7 | 1 | 74 | 121 | 38 | 39 |
| | 2 | 51 | 87 | 37 | |
| | 3 | 76 | 97 | 44 | |
| | 4 | 74 | 103 | 42 | |
| | 5 | 82 | 144 | 36 | |
| A:A2/B:B #12 | 1 | 54 | 72 | 43 | 41 |
| | 2 | 64 | 91 | 41 | |
| | 3 | 64 | 80 | 44 | |
| | 4 | 60 | 100 | 37 | |
| | 5 | 72 | 106 | 40 | |
| A:A1/B:B | 1 | 36 | 117 | 23 | 24 |
| | 2 | 30 | 110 | 27 | |
| | 3 | 20 | 93 | 22 | |
| | 4 | 22 | 97 | 23 | |
| | 5 | 35 | 133 | 26 | |

**Table 3.2: Double mutant confirmation data.** Double mutant data collected from cross shown in Appendix. Both strains of chimera A:$A_2$/B:B are double *unc-54;unc-45* mutants, while chimera A:$A_1$/B:B is only a single *unc-54* mutant.

# 4. Discussion

## 4.1. Difficulties in making an UNC-45 antibody

As mentioned previously, our lab had made a polyclonal antibody in rabbits to GST::UNC-45(18-76). This fragment of UNC-45 contains the amino terminal TPR repeats. The serum from the rabbit with the best response, 7N5, was purified on a GST::UNC-45(18-76) column and used for immunostaining (Ao and Pilgrim, 2000). When I came into the lab, the antibody had not been used for 4 months and was stored at 4°C. After many immunostaining attempts with this aliquot of antibody, as well as antibody purified from serum that had been stored at –80°C, I was not able to get a specific signal. The serum had lost its specificity during storage at –80°C so I began producing a new UNC-45 antibody.

I used the same method used to produce the 7N5 antibody on two different sets of rabbits; however, no UNC-45 specific serum was obtained. Attempts at monoclonal antibodies were also made in order to get a more stable antibody that could be easily re-purified. After two failed attempts of monoclonal production I decided to try and manipulate the sera I had already obtained and try to increase their specificity. My first theory was that the GST antibodies present in the sera were overwhelming the UNC-45 antibodies, causing a low UNC-45 specificity. After determining that excess GST antibodies were not the problem I focused on the composition of the GST::UNC-45(18-76) fusion protein.

For one last attempt to increase the specificity of my antibody I decided to make a column that contained the UNC-45(18-76) fragment cleaved from GST. I thought that since the UNC-45 fragment was so small and GST makes up 75% of the protein, the

UNC-45 epitopes the rabbit had generated antibodies against were being masked by the way GST was folding. This would block the UNC-45 epitopes on the column so the UNC-45 antibodies running over the column had nothing to bind to and just flowed through. Thus, when the bound antibodies were purified from the column no UNC-45 antibodies were present. I hoped that by physically separating the GST from UNC-45(18-76) and binding both to the column, the UNC-45 epitopes that were once hidden would now be able to bind the UNC-45 antibodies present in the serum running over the column. Elution of the column should release some UNC-45 specific antibodies.

I attempted this procedure on all the sera and did get a specific UNC-45 signal. I concluded that the small size of UNC-45(18-76) had been the problem hindering my ability to make the antibody. Perhaps if a larger fragment of UNC-45 were used then the same problem could be avoided and the UNC-45 epitopes would not be masked by GST. The best response was with rabbit 4E1 so this was used for subsequent immunostaining.

### 4.2. Significance of UNC-45 binding to the rod of MHC B

My results show that it is the rod region of MHC B that is required for UNC-45 localization to the thick filament. It was originally hypothesized that the head of MHC B would be required to localize UNC-45 properly (Barral et al., 2002). The myosin head has a complex three-dimensional structure and needs the help of chaperones to achieve proper conformation. When UNC-45 was shown to localize to the MHC B region of the thick filament (Ao and Pilgrim, 2000) and shown to have chaperone properties (Barral et al., 2002) it was suggested that it could be acting as a muscle specific chaperone/co-chaperone to help fold the myosin head. Through *in vitro* experiments, it was also shown that UNC-45 interacted with an S1 myosin fragment from scallop, which contains the head domain (Barral et al., 2002). The data presented in this thesis examines the region of MHC B required for UNC-45 binding in an *in vivo* assay as well as the requirement for UNC-45 in chimeric myosins. The work was all done in *C. elegans*, using native *C. elegans* myosins, instead of a fragment of myosin from scallop, to more directly examine the relationship between UNC-45 and MHC B.

As mentioned above, the chimeric myosins B:A:A, B:A:B, B:A:Δ, and A:$A_1$/B:B did not localize UNC-45 to the thick filament. Three of these chimeric myosins contain the MHC B head, which is where UNC-45 was expected to interact. These data conflict with the simplest interpretation of the *in vitro* data that found the S1 fragment (containing the head domain) directly interacted with UNC-45. The chimeric myosins A:$A_2$/B:B and B:B:A did localize UNC-45 to the thick filament, indicating they had a common MHC B region that UNC-45 bound to. The only MHC B region these two chimeric myosins have in common is the carboxyl half of the rod, which indicates that is the region required for bringing UNC-45 into contact with MHC B. Contradicting this conclusion, chimera A:B:B did not show proper localization of UNC-45, even though it had the carboxyl half of the MHC B rod. However, as discussed I believe this strain has been misnamed and that A:B:B is not the chimeric myosin present in the transgenic worms. The epitope for DM 5-8 (MHC B) is present in the carboxyl half of the rod so the myosins in these worms should be able to stain with DM 5-8, as do all the other chimeras containing the portion of MHC B. DM 5-8 staining in these worms was undetectable, identical to the chimeric myosins that were missing the epitope. From this observation, these worms could not contain the A:B:B chimera so I excluded the data from them. (Note: when these strains were sent, Dr. Hoppe was also unsure whether it had been correctly labeled.)

This data now suggests that there could be two UNC-45 binding sites on the MHC B molecule: one on the head where UNC-45 exerts its chaperone activity (as shown by Barral et al., 2002) and the one I found on the rod used to localize it permanently to the thick filament in adult worms.

### *4.2.1. UNC-45 chaperone activity during muscle formation*

Although UNC-45 binds to MHC B in the mature filaments (Ao and Pilgrim, 2000), it is likely acting as a chaperone before the filaments are even assembled. The myosin head has a complex structure and requires the use of chaperones to help complete folding before it can be assembled into the thick filament. UNC-45 is a muscle specific protein that binds Hsp90 and together they could be working as a chaperone complex to help fold the myosin. For this process, UNC-45 does not need to be directly localized to the thick filament, as folding at this stage takes place in the cytoplasm. UNC-45's involvement at this stage of folding is supported by UNC-45 localization in L1 larval worms (Ao and Pilgrim, 2000). Immunostaining against UNC-45 showed that while MHC B and MHC A were already incorporated into the filament, UNC-45 was still diffuse in the cytoplasm (Figure 1.10). UNC-45 likely remains diffuse in the cytoplasm, acting with Hsp90 to help fold the myosin heads, until the filaments are fully formed. At this point Hsp90 is no longer needed as it only associated with folding intermediates (Srikakulam and Winkelmann, 2003) and UNC-45 becomes localized to the thick filament to perform its secondary role of chaperone monitoring (see below)

### *4.2.2. UNC-45 chaperone monitoring activity in adult worms*

The myosin head is the motor domain of the molecule, used to generate force. The process is ATP dependent, requires both direct interactions of the myosin head and actin, and a change in the conformation of the head. Through each contraction-relaxation cycle a huge amount of stress is put on the myosin head, which could negatively impact its conformation. It is possible that UNC-45 has taken on the role of monitoring the shape of the myosin head during the contraction-relaxation cycles and is able to act as a chaperone to re-fold any heads that become denatured. It could also be acting a co-chaperone whose main role would be to monitor the folding state of the head and then recruit Hsp90 and Hsp70 to help with the actual folding. The lateral arms of the myosin thick filament (composed of MHC B) are what interact with actin so it is expected that the MHC B heads will be undergoing a lot more stress than the MHC A heads (which are only found in the central region) and consequently will require more chaperone monitoring.

In wildtype worms, UNC-45 appears to remain bound to MHC B in the thick filament through out the life of the adult worm. This system of UNC-45 remaining bound to the mature myosin to monitor and provide chaperone activity is unique. All chaperones characterized to date help fold their substrates in the cytoplasm and then once the protein is in the mature form they continue to the next unfolded molecule. Most of the other UCS homologs (except Rng3) do not associate with their myosins after initial folding in the cytoplasm, making UNC-45 not only unique among chaperones but also among its family of myosin chaperones. Hsp90 has not yet been detected in the mature thick filaments so it appears that UNC-45 could possess enough chaperone activity to act as a full chaperone, instead of just a co-chaperone for Hsp90 as is the case in the cytoplasm during muscle formation. It is also possible that UNC-45 still recruits Hsp90 to the thick filament through its TPR domain and the tethering mechanism brings Hsp90 into contact with the MHC B head. Spatially, this would be more probable since the domain where UNC-45 binds in the rod is a long distance from the head. By making a bridge between UNC-45, Hsp90, and the MHC B head the chaperone activities could easily be directly by UNC-45 and carried out by both UNC-45 and Hsp90. This interaction might only occur when the head needs to be re-folded so it would be hard to see this by immuno localization studies.

As mentioned above, the myosin head is undergoing a number of complex protein-protein interactions to generate force. If UNC-45 remains bound to the thick filament to monitor the conformation of the MHC B head it cannot interfere with all the processes going on during the contraction-relaxation cycle. If UNC-45 binds to the MHC B head and remains there it could end up hindering the function of the motor domain, which would be detrimental to the muscle system. My data suggests UNC-45 avoids this problem by binding to the rod of MHC B, thereby leaving the head unencumbered. By binding to the rod, UNC-45 is still in close proximity to monitor and exert chaperone activity when needed but does not interfere with the motor domain.

Another possibility is that UNC-45 only interacts with denatured myosin heads, which is supported by the data found in Barral et al., (2002). UNC-45 could be bound/tethered to the rod in such a way that it comes into contact with the myosin head during each muscle contraction. UNC-45 would only recognize unfolded or denatured regions (perhaps at a particular unstable part of the head) and bind to them to either exert chaperone activities or recruit Hsp90. This method would again leave the head free to perform its work when it is correctly folded and functional.

### 4.3. UNC-45 localization is separate from function

Once I had determined that UNC-45 was cytoplasmically localized in worms containing chimeras B:A:A, B:A:B, B:A:Δ, and A:$A_1$/B:B, I was surprised to observe that these worms were capable of movement. Quantitatively, for these chimeric myosins, there appears to be no difference in movement of worms in which UNC-45 is correctly localized to the thick filament and when UNC-45 is cytoplasmic. Since an *unc-45(ts)* worm grown at the restrictive temperature is completely paralyzed with disorganized thick filaments we know that UNC-45 is playing a key role in thick filament assembly. My data suggests that UNC-45 does not have to bind to the thick filament to perform this function, in fact, its major role could be when UNC-45 is in the cytoplasm, not at the thick filaments. If UNC-45 is acting as a chaperone (in partner with Hsp90) to help fold the MHC B head, cytoplasmic localization and function would not be surprising. As described above, most chaperones are cytoplasmic in nature and do not associate with the mature form of their substrate. The observation that worms can still move when UNC-45 is cytoplasmic suggests that this could be the major role of UNC-45 and localization to the thick filament is a secondary role. From this data, it appears as though the localization of UNC-45 and the primary function of UNC-45 have, for the fist time, been separated.

Even though UNC-45's initial chaperone role is cytoplasmic the fact that UNC-45 remains bound to the thick filament throughout adult life suggests that it does play a role there. As described above, this role is hypothesized to be chaperone monitoring of the

MHC B heads during muscle contraction. It appears now that this process is not essential to the worm, at least in the laboratory environment. The physical stresses a worm goes through in the laboratory are much smaller that what they would encounter in the wild. If the muscle system does not undergo a lot of stress, the MHC B heads can maintain their conformation and there would be no need for UNC-45 to re-fold them. This is what the worms encounter in the laboratory environment so as long as UNC-45 can fold the myosin heads in the cytoplasm the absence of UNC-45 at the thick filament would not affect their movement. The MHC B heads may not undergo enough stress in the lab to cause them to mis-fold. The chaperone monitoring system of UNC-45 would only be vital if the worms were exposed to heightened physical stress and/or temperature, which would occur in their natural environment. Increased movement and stress would cause the muscle system to work much harder, forcing the components to move faster. It would be in this situation that the MHC B heads could start coming unfolded and the function of UNC-45 would be essential to maintain them in a state competent for interaction with the thin filaments. If worms containing the chimeric myosins unable to localize UNC-45 to the thick filament were exposed to a heightened stress environment their thick filament organization may slowly break down. They would eventually become paralyzed because UNC-45 would not be able to repair the conformation of the mis-folded MHC B heads.

The chimeric myosins expressed in the worms are from an extrachromosomal array, in which proteins are highly over-expressed compared to wild-type levels. It is also possible that UNC-45 localization to the thick filament is essential in laboratory worms but due to the sheer amount of myosin available to make the thick filament, the amount that becomes mis-folded does not have an impact on the function of the filament.

If the expression levels of the chimeric myosins were reduced to the same as wildtype it is possible that a defect in movement or filament organization could be seen when UNC-45 is mislocalized. There would not be an excess of myosin present to substitute for the mis-folded ones and the filament would slowly break down.

The fact that the worms containing mis-localized UNC-45 were still able to move has also shed light on what could be happening in the *unc-45(ts)* mutants. In these mutants UNC-45 is still associated with the disorganized thick filament so the mutation has not disrupted the binding of UNC-45 to MHC B. It is possible that the mutation in *unc-45(ts)* worms has disrupted the cytoplasmic chaperone activity of UNC-45. If this activity was disrupted the MHC B heads would never properly fold when the thick filament is initially being laid down, leading to the disorganization seen in these worms. It would not matter if UNC-45 could still localize to the thick filament because the disorganization would be too severe to fix. Since this mutation leads to paralysis of worms while mis-localization appears to have no effect in the laboratory it would suggest that the cytoplasmic function is much more critical than the thick filament association.

### 4.4. Differential requirements for UNC-45 in assembly of the chimeric thick filament

In addition to determining what region of MHC B is required for UNC-45 localization, the chimeric myosins can also help distinguish the regions of MHC B and even MHC A that require (or could require) UNC-45 activity. By crossing the worms carrying the chimeric myosins (in an *unc-54(0)* background) into *unc-45(ts)* worms and growing them at the restrictive temperature we were able to determine whether the chimeric myosins can assemble in the absence of UNC-45. If the double mutants are paralyzed the chimeric myosins must require UNC-45 in order to assemble a proper thick filament, whereas if the double mutants are able to move, then the chimeric thick filament does not require fully functional UNC-45. These results will help resolve what region of MHC B requires UNC-45 to fold and assemble and could possibly even show if a region of MHC A is dependent on UNC-45 function.

Due to time constraints, I was only able to confirm two chimera-containing double mutants. Chimera A:$A_2$/B:B was able to assemble a functional thick filament in the absence of UNC-45, which was determined by the ability of the worms to move. Chimera B:A:Δ was not able to assemble a functional thick filament in the absence of UNC-45 so consequently the double mutants were paralyzed. I also assumed that Chimera B:B:B would also be paralyzed in a double mutant, even though I could not obtain the double mutant due to the extremely high transmission rate of the chimera. This double mutant would be essentially acting as a *unc-45(ts)* single mutant (which we know is paralyzed), as the chimeric myosin is full length MHC B, with no MHC A sequence.

Chimera A:$A_2$/B:B contains the MHC A head and the first bit of the rod. The rest of the rod and the tail is composed of MHC B. The evidence that this chimera can assemble well enough to allow movement indicates that the MHC A head does not require UNC-45 to help it assemble. Somehow the two isoforms have diverged in function in that the MHC B head requires a chaperone to help it fold whereas the MHC A head does not. This is very intriguing because the two isoforms are quite similar (with 77% similarity and 67% identity at the amino acid level) so it is unclear as to how the structure of the MHC A head is different enough from the MHC B head to allow it to fold without the help of UNC-45. It could be due to the fact that the MHC B isoform makes up the majority of the thick filament and so interacts with actin at a much higher frequency than MHC A. It would therefore be doing much more work than MHC A, increasing the possibility of its heads becoming misfolded and needing to be fixed. These data also suggests that if UNC-45 has a role in helping MHC A assemble (as has been hypothesized due to their similar "pat" phenotype) it is probably the cytoplasmic function of UNC-45 that occurs during the very early assembly/initiation of the thick filament.

The presence of the MHC B head in chimera B:A:Δ further supports the requirement of UNC-45 for the folding of the MHC B head (cytoplasmic and/or thick filament associated activity). This chimera is unable to assemble a functional thick filament in the absence of UNC-45, resulting in paralyzed worms. We already knew that full length MHC B could not assemble in the absence of UNC-45 but did not know if there was a particular region that was dependent on UNC-45 or whether it was the entire MHC B molecule. We can now conclude that the MHC B head in particular requires UNC-45 function. The MHC B head has developed a dependency on UNC-45 to help it fold and without it the myosin molecules cannot be assembled and maintained in a functional filament.

Chimera B:A:Δ is also missing the entire tail region of the chimeric myosin so at this point it cannot be differentiated whether it is the presence of the MHC B head or the absence of a tail that is resulting in paralyzed double mutants. In addition to Chimera B:A:Δ missing the tail region it is also missing four amino acids from the C-terminal end of the coiled-coil rod. These four residues, studied originally in MHC A (Hoppe et al., 2003), are conserved in a variety of organisms, with the two charged residues (lysine and arginine) having a very high level of conservation. This suggests that these four residues play an important role in the coiled-coil rod of striated muscle. In MHC A, the four residue sequence is KIRA and in MHC B it is SIRA, with both containing the conserved lysine and arginine. The importance of these four residues was determined when B:A:Δ (Δ34in the Hoppe et al., 2003 paper) and a new chimera (only missing the tail region (Δ30)) were examined for the ability to rescue the lethality of a double *unc-54(0); myo-3(0)* mutant. The chimera that was only missing the tail (Δ30)) was able to rescue the lethality and is thus competent to function as the sole myosin heavy chain whereas B:A:Δ (Δ34) was unable to rescue the lethality (Hoppe et al., 2003). Therefore, the four residues of the coiled-coil rod that are present in Δ30 but absent in Δ34 are essential for some aspect of myosin function (Hoppe et al., 2003). Worms expressing Δ34 as their sole myosin arrest elongation at the two-fold stage of embryogenesis and die as misshapen L1 larvae, which is very similar to the "pat" phenotype seen in *myo-3(0)* and *unc-45(0)* worms. Immunostaining revealed that Δ34 showed delayed localization of the truncated myosin into the discrete bands that form at the beginning of thick filament assembly. The Δ34 remained diffuse in the cytoplasm whereas Δ30 localized properly indicating the KIRA sequence is required for timely localization of MHC A to initiate thick filament assembly. Since worms containing B:A:Δ (Δ34) in a double *unc-54(0); unc-45(ts)* mutant are paralyzed it is possible that UNC-45 interacts with the tail domain or the four residue region of the rod (SIRA in MHC B). Although it hasn't been examined, the SIRA region in MHC B could also be playing a crucial role in the initial assembly of MHC B into the thick filament. The rod directs the MHC B molecules to form the thick filament and the SIRA residues could have a major role in assisting with assembly. This could occur through an interaction with UNC-45 that is disrupted in *unc-45(r450);unc-54(0)* B:A:Δ(Δ34) due to the absence of SIRA, resulting in altered assembly of the thick filament and paralysis of the worms. It is possible that UNC-45 could be involved in both processes described above: proper folding of the MHC B head and helping the rod region assemble into the thick filament through interactions with the C-terminal end of the rod or the non-helical tail.

### 4.5. Future directions

#### *4.5.1. Determining whether UNC-45 is required in the remaining chimeras*

Double mutants of the remaining chimeras need to be obtained to determine whether it is the presence of the MHC B head or the loss of the tail/SIRA residues in B:A:Δ that results in paralysis. B:A:A would be an important one to test to differentiate between the head and tail regions. If B:A:A is paralyzed it would suggest that the MHC B head does require UNC-45 activity to properly fold. If it is found that the tail region requires UNC-45 it would be useful to obtain the Δ30 chimera and cross it into an *unc-45(ts)* background. If this double mutant was not paralyzed that would suggest the four conserved coiled-coil residues absent in Δ34 have an important interaction or requirement for UNC-45. The results of these double mutant experiments will help determine what region(s) of MHC B requires UNC-45. Although we have shown that UNC-45 localizes to the rod region, I believe it will be found that the head region of MHC B requires UNC-45 in order to fold and function. Even though UNC-45 does not localize to the head it can still exert its chaperone activities by a tethering mechanism formed on the rod of the MHC B molecule and will bind and be associated with the head at various times during the life of the worm.

It is also interesting to note that the worms carrying the chimeric myosins in which UNC-45 is mis-localized are able to move. This would not present a problem when the thick filaments are initially assembling because it is predicted that UNC-45 functions in the cytoplasm as a chaperone so it would not need to localize directly to the thick filament. However, when the MHC B heads are undergoing a lot of stress UNC-45 is required to help maintain the myosin heads in the correct conformation. If this is true I would expect that when the worms that contain mis-localized UNC-45 are thermally or mechanically stressed they would start to show some movement defects due to the inability of UNC-45 to help re-fold the MHC B heads. Over time, if movement was compared under stress with worms in which UNC-45 can localize it is possible that worms that mislocalize UNC-45 would have much slower movement and potentially become paralyzed. Stress could be achieved by growing them in liquid media or at a higher temperature where their muscles have to work harder. If immuno-staining is performed against MHC B when the worms are grown under more stressful conditions, the thick filament would likely become more disorganized. Although I could not determine a difference quantitatively, motility tests should also be done to confirm if there is a slight defect that is undetectable by the naked eye. By comparing the movement of wild-type, correctly localized chimeric worms, and mis-localized chimeric worms there may be a noticeable difference in reduced mobility of the worms that contain mis-localized UNC-45.

### *4.5.2. Direct evidence for UNC-45 acting as a chaperone on MHC B heads*

So far, the only evidence that UNC-45 is performing chaperone activities on the MHC B head is the *in vitro* data in which UNC-45 bound the S1 fragment and it displayed chaperone activities on sample substrates (not myosin). Direct evidence of this role of UNC-45 could be obtained by the use of conformation sensitive antibodies that react to myosin (specifically MHC B). If these antibodies were used on *unc-45(ts)* mutants grown at the restrictive temperature it could be determined which (if any) regions of myosin are unfolded. These antibodies will only bind to an epitope exposed on a mis-folded myosin head and once the head is properly folded the epitope is hidden so staining can no longer be detected. If UNC-45 is working as a chaperone for the MHC B head we would expect that the antibody that recognizes an epitope when the head is mis-folded would be picked up in an *unc-45* mutant.

### *4.5.3. Examine the role of UNC-45 in the assembly of MHC A*

Even though an interaction between MHC A and UNC-45 has not been demonstrated (*in vitro* or *in vivo),* it has been thought that UNC-45 could interact with MHC A very early in muscle development to initiate thick filament assembly. MHC A and UNC-45 null mutants both have a similar "pat" phenotype and genetic evidence suggests that the two interact. If UNC-45 was helping MHC A initiate filament assembly this would occur during the very early stages of muscle development. In the L1 larval stage, UNC-45 is still cytoplasmic while MHC B has been assembled into the thick filament and UNC-45 is still acting on MHC B. UNC-45's role in MHC A filament initiation might only occur in the cytoplasm and the two proteins never have to directly interact in the thick filament. If the two proteins do interact a possible interaction site in MHC A could be the KIRA residues. These residues are essential for MHC A initiation of the filament so they are a likely candidate for UNC-45 interaction. This interaction could occur for just a very brief time during early muscle development so it might be difficult to detect on immunostaining co-localization studies.

It has been difficult to determine if UNC-45 is playing a role in filament initiation due to the maternal contribution of UNC-45 (described in Section 1.5.2). In *unc-45* lethal mutants, MHC A assembly is observed to initiate (Venolia and Waterston, 1990), but this could be carried out by the maternal contribution of UNC-45. If the maternal UNC-45 was removed and MHC A initiation could not occur then this would indicate that UNC-45 does have a role with MHC A in filament initiation, most likely through a cytoplasmic interaction.

Immunoprecipitation would also be a good method to examine the interactions of UNC-45. Co-immunoprecipitation was performed on UNC-45 and the S1 fragment of a myosin from scallop, indicating UNC-45 does indeed interact with myosins (Barral et al., 2002). It has not yet been shown directly that UNC-45 interacts with *C. elegans* MHC B or MHC A. It would be expected that UNC-45 would pull down MHC B, as well as its other co-chaperones, Hsp90 and Hsp70. If UNC-45 also pulled down MHC A it would support a direct role for UNC-45 in filament initiation and not just assembly after the fact. Now that an UNC-45 antibody has been produced these co-immunoprecipitation experiments should be relatively straightforward.

### *4.5.4. Determine which regions of UNC-45 can rescue unc- 45(ts) mutants*

I was also unable to inject the various deletion constructs of UNC-45 into *unc-45(ts)* animals and determine which region of UNC-45 is required for thick filament assembly. Since UNC-45 has roles in embryonic polarity as well as thick filament assembly I would think that each domain could be serving a different purpose. I would expect that the UCS domain would be able to restore thick filament assembly and motility to *unc-45(ts)* worms. The UCS domain contains the chaperone activities, which would be required for MHC B assembly. The TPR domain by itself would probably not be able to rescue the worms. Its main function is to bring Hsp90 and Hsp70 into contact with the thick filament, but without the UCS domain it would be unlikely that just these two proteins would be able to assemble the thick filament. Since the Central domain has no known function but is highly conserved across metazoans I would predict that this domain is very important to UNC-45 and could potentially rescue the defects of an *unc-45(ts)* mutant. These experiments would again be straightforward because all but one of the constructs are made and they just have be injected into *unc-45(ts)* worms then once a stable line is obtained they need to be transferred to the restrictive temperature and scored for rescue.

When these rescuing studies are being done they will also show which region of UNC-45 is required for the localization to the thick filament. Since the constructs are fused in frame to GFP the localization of GFP, either cytoplasmic or thick filament associated, will indicate which region of UNC-45 localizes it to the thick filament. I expect that the Central or UCS domain will be required due to the *in vitro* data found by

Barral et al (2002) that showed the TRP(-) fragment could bind myosin, indicating it is not involved in myosin binding. Since the UCS domain is where the chaperone activities lie I believe it will be the Central domain that is required to bind UNC-45 to myosin so the UCS domain is free to perform its essential function.

## 5. Appendix

Strains and genotypes

*C. elegans*

| | |
|---|---|
| N2 | wild-type, Bristol isolate |
| DR176* | *unc-54(e190)I; eDp23 V* |
| CB190* | *unc-54(e190)I* |
| DP32 | *unc-45(r450)* |
| DP396 | *unc-45(r450);him-8* |
| DP397 | *unc-54(e190)* stEx85*;unc-45(r450)* |
| DP398 | *unc-54(e190)* stEx85*; unc-45(450);him-8* |
| DP399 | *unc-54(e190)* stEx153*; unc-45(r450)* |

Strains carrying the chimeric myosins are listed in Table 3.1

*Strains were provided by the *Caenorhabditis* Genetics Center (Minneapolis, MN)

## 6. References

Agashe, V. and F.U. Hartl. 2000. Roles of molecular chaperones in cytoplasmic protein folding. *Cell and Development Biology* **11:** 15-25

Ao, W. 2001. Ph.D. Thesis. *Caenorhabditis elegans* UNC-45 defines a novel class of myosin-associated proteins. Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

Ao, W. and D. Pilgrim. 2000. *Caenorhabditis elegans* UNC-45 is a component of muscle thick filaments and co-localizes with myosin heavy chain B, but not myosin heavy chain A. *Journal of Cell Biology.* **148**: 357-384

Barral, J.M., C.C. Bauer, I. Oritz, and H.F. Epstein. 1998. Unc-45 mutations in *Caenorhabditis elegans* implicate a CRO1/She4p-llike domain in myosin assembly. *Journal of Cell Biology* **143:** 1215-1225

Barral, J. and H. Epstein. 1999. Protein machines and self assembly in muscle organization. *BioEssays* **21:** 813-823

Barral, J.M., A.H. Hutagalung, A. Brinker, F.U. Hartl, and H.F. Epstein. 2002. Role of the myosin assembly protein UNC-45 as a molecular chaperone for myosin. *Science* **295:** 669-671

Bejsovec, A. and P. Anderson. 1988. Myosin heavy-chain mutations that disrupt *Caenorhabditis elegans* thick filament assembly. *Genes and Development* **2:** 1307-1317

Bejsovec, A. and P. Anderson. 1990. Functions of the myosin ATP and actin binding sites are required for *C. elegans* thick filament assembly. *Cell* **60:** 133-140

Berteaux-Lecellier, V., D. Zickler, R. Debuchy, A. Panvier-Adoutte, C. Thompson-Coffe, and M. Picard. 1998. A homologue of the yeast SHE4 gene is essential for the transition between the syncytial and cellular stages during sexual reproduction of the fungus *Podospora anserina. EMBO J* **17:** 1248-1258

Blagosklonny, M.V., G.S. Wu, S. Omura, and W.S. el-Deiry. 1996. Proteasome-dependant regulation of p21/WAF1/CIP1 expression. *Biochemical and Biophysical Research Communications* **227:** 564-569

Blatch and Lassle. 1999. The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *BioEssays* **21:** 932-939

Bonnemann, C.G. and N.G. Laing. 2004. Myopathies resulting from mutations in sarcomeric proteins. *Current Opinions in Neurology* **17:** 527-537

Brenner, S. 1973. The genetics of behavior. *British Medical Bulletin* **29:** 269-271

Brenner, S. 1974. The genetics of *Caenorhabditis elegans. Genetics.* **77**: 71-94

Buchner, J. 1999. Hsp90 and Co. – a holding for folding. *Trends in Biochemical Sciences* **24:** 136-141

Burr, AH, C Gans. 1998. Mechanical significance of obliquely striated architecture in nematode muscle. *Biology Bulletin* **194:** 1-6

Caplan, A.J. 1999. Hsp90's secrets unfold: new insights from structural and functional studies. *Trends in Cell Biology* **9:** 262-268

Castellani, L., B.W. Elliott, Jr., and C. Cohen. 1988. Phosphorylatable serine residues are located in a non-helical tailpiece of a catch muscle myosin. *Journal of Muscle Cell Research and Cell Motility* **9:** 533-540

Chadli, A., I. Bouhouche, W. Sullivan, B. Stensgard, N. McMahon, and M.G. Catelli. 2000. Dimerization and N-terminal domain proximity underlie the function of the molecular chaperone heat shock protein 90. *PNAS* **97:** 12524-12529

Chen, S., V. Prapapanich, R.A. Rimerman, B. Honore, and D.F. Smith. 1996. Interactions of p60, a mediator of progesterone receptor assembly, with heat shock proteins hsp90 and hsp70. *Molecular Endocrinology* **10:** 682-693

Chen, S. and D. Smith. 1998. Hop as an adaptor in the heat shock protein 70 (hsp70) and hsp90 chaperone machinery. *Journal of Biological Chemistry* **273:** 35194-35200

Cheney R.E. and M.S. Mooseker. 1992. Unconventional myosins. *Current Opinions in Cell Biology* **4:** 27-35

Chow, D., R. Srikakulam, Y. Chen, and D.A. Winkelmann. 2002. Folding of the striated muscle myosin motor domain. *Journal of Biological Chemistry.* **277:** 36799-36807

Cohen, C, D.E. Lanar, and D.A.D Parry. 1987. Amino acid sequence and structural repeats in schistosome paramyosin match those of myosin. *Bioscience Reports* **7:** 11-16

Connell, P., C.A. Ballinger, J. Jiang, Y. Wu, L.J. Thompson, J. Hohfeld, and C. Patterson. 2001. The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nature: Cell Biology* **3:** 93-96

Corsi, A.K., S.A. Kostas, A. Fire, and M. Krause. 2000. *C. elegans* twist plays and essential role in non-striated muscle development. *Development* **127:** 2041-2051

Cyr, D.M., J. Hohfeld, and C. Patterson. 2002. Protein quality control: U-box containing E3 ubiquitin ligases join the fold. *Trends in Biochemistry Science* **27:** 368-375

Dibb, N.J., D.M. Brown, J. Karn, D.G. Moerman, S.L. Bolten, and R.H. Waterston. 1985. Sequence analysis of mutations that affect the synthesis, assembly and enzymatic activity of the *unc-54* myosin heavy chain of *Caenorhabditis elegans*. *Journal of Molecular Biology* **183:** 543-551

Dibb, N.J., I.N Maruyama, M. Krause, and J. Karn. 1989. Sequence analysis of the complete *C. elegans* myosin heavy chain gene family. *Journal of Molecular Biology* **205:** 603-613

Epstein, H.F., and D. Fischman. 1991. Molecular analysis of protein assembly in muscle development. *Science* **251:** 1039-1044

Epstein, H.F. and J.N. Thomson. 1974. Temperature-sensitive mutation affecting myofilament assembly in *C. elegans*. *Nature* **250:** 579-580

Epstein, H.F., R.H. Waterston, and S. Brenner. 1974. A mutant affecting the heavy chain of myosin in *Caenorhabditis elegans*. *Journal of Molecular Biology* **90:** 291-300

Epstein, H.F., I. Ortiz, and L.A.T. Mckinnon. 1986. The alteration of myosin compartmentalization in specific mutants of *Caenorhabditis elegans*. *Journal of Cell Biology* **103:** 985-993

Epstein, H.F., D.M. Miller, I. Ortiz, and M.F. Schmid. 1995. Preliminary three-dimensional model for nematode thick filament core. *Journal of Cell Biology*. **100:** 904-915

Etheridge, L., P. Diiorio, and C.G. Sagerström, 2002. A Zebrafish unc-45-related gene expressed during muscle development. *Developmental Dynamics* **224**: 457-460.

Finney, M., and G. Ruvkan. 1990. The *unc-86* gene product couples cell lineage and cell identity in *C. elegans*. *Cell*. **63:** 895-905.

Franscis, G.R. and R.H. Waterston. 1985. Muscle organization in *Caenorhabditis elegans*: localization of proteins implicated in thin filament attachment and I-band organization. *Journal of Cell Biology* **101:** 1532-1549

Gordon, A.M., E. Homsher, and M. Regnier. 2000. Regulation of contraction in striated muscle. *Physiological Reviews* **80:** 853-924

Gregorio, C.C., and P.B. Antin. 2000. To the heart of myofibril assembly. *Trends in Cell Biology* **10:** 355-362

Guo, S. and K.J. Kemphues. 1996. A non-muscle myosin required for embryonic polarity in *Caenorhabditis elegans*. *Nature* **382:** 455-458

Harfe, B.D., A. Vaz Gomes, C. Kenyon, J. Liu, M. Krause, and A. Fire. 1998b. Analysis of a *C. elegans* twist homolog identified conserved and divergent aspects of mesodermal patterning. *Genes and Development* **12:** 2623-2635

Hartl, F. U. and M. Hayer-Hartl. 2002. Molecular chaperones in the cytosol. *Science* **295**: 1852-1858

Hoppe, P.E. and R.H. Waterston. 1996. Hydrophobicity variations along the surface of the coiled-coil rod may mediate striated muscle myosin assembly in *Caenorhabditis elegans*. *The Journal of Cell Biology*. **135:** 371-382

Hoppe, P.E., R.C. Andrews, and P.D. Parikh. 2003. Differential requirements for the nonhelical tailpiece and the C terminus of the myosin rod in *Caenorhabditis elegans* muscle. *Molecular Biology of the Cell* **14:** 1677-1690

Hoppe, T., G. Cassata, J.M. Barral, W. Springer, A.H. Hutagalung, H.F. Epstein, and R. Baumeister. 2004. Regulation of the myosin-directed chaperone UNC-45 by a novel E3/E4-multiubiquitylation complex in *C. elegans*. *Cell* **118:** 337-349

Hutagalung, A. H., M.L. Landsverk, M.G. Price, and H.F. Epstein. 2002. The UCS family of myosin chaperones. *Journal of Cell Science* **115**: 3983-3990

Imai, Y., M. Soda, S. Hatakeyama, T. Akagi, T. Hashikawa, K.I. Nakayama, and R. Takahashi. 2002. CHIP is associated with Parkin, a gene responsible for familial parkinson's diseases, and enhances its ubiquitin ligase activity. *Molecular Cell* **10:** 55-67

Jakob, U., H. Lilie, I. Meyer, and J. Buchner. 1995. Transient interaction of Hsp90 with early unfolding intermediates of citrate synthases. Implications for heat shock in vivo. *Journal of Biological Chemistry* **270:** 7288-7294

Jascur, T., H. Brickner, I. Salles-Passador, V. Barbier, A. El Khissiin, B. Smith, R. Fotedar, and A. Fotedar. 2005. Regulation of p21$^{WAF1/CIP1}$ stability by WISp39, a Hsp90 binding TPR protein. *Molecular Cell* **17:** 237-249

Kachur, T, W. Ao, J. Berger, and D. Pilgrim. 2004. Maternal UNC-45 is involved in cytokinesis and co-localizes with non-muscle myosin in the early *Caenorhabditis elegans* embryo. *Journal of Cell Science* **117:** 5313-5321

Karn, J., S. Brenner, and L. Barnett. 1983. Protein structural domains in the *C. elegans unc-54* myosin heavy chain gene are not separated by introns. *PNAS* **80:** 4253-4257

Koegl, M., T. Hoppe, S. Schlenker, H.D. Ulrich, T.U. Mayer, and S. Jentsch. 1999. A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* **96:** 635-644

Kriwacki, R.W., L. Hengst, L. Tennant, S.I. Reed, and P.E. Wright. 1996. Structural studies of p21Waf1/Cip1/Sdi1 in the free and Cdk-2 bound state: conformational disorder mediates binding diversity. *PNAS* **93:** 11504-11509

Lassle, M., G.L. Blatch, V. Kundra, T. Takatori, and B.R. Zetter. 1997. Stress-inducible, murine protein mST11. Characterization of binding domains for heat shock proteins and *in vitro* phosphorylation by different kinases. *Journal of Biological Chemistry* **272:** 1876-1884

Li, C. and M. Chalfie. 1990. Organogenesis in *C. elegans*: positioning of neurons and muscles in the egg-laying system. *Neuron* **4:** 681-695

Liu F, C.C. Bauer, I. Ortiz, RG. Cook, MF. Schmid, HF. Epstein. 1998. β-filagenin, a newly identified protein coassembling with myosin and paramyosin in *Caenorhabditis elegans*. *Journal of Cell Biology* **140:** 347-353

Liu F, I Ortiz, A. Hutagalung, C. Bauer, R. Cook and H.F. Epstein. 2000. Differential assembly of α– and γ–filagenins into thick filaments in *Caenorhabditis elegans*. *Journal of Cell Science* **113:** 4001-4012

Long, R.M., R.H. Singer, X. Meng, I. Gonzalez, K. Nasmyth, and R.P Jansen. 1997. Mating type switching in yeast controlled by asymmetric localization of ASH1 mRNA. *Science* **277:** 383-387

Lord, M. and T.D. Pollard. 2004. UCS protein Rng3 activates actin filament gliding by fission yeast myosin II. *Journal of Cell Biology* **167:** 315-325

Mackenzie, J. and H.F. Epstein. 1980. Paramyosin in necessary for determination of nematode thick filament length *in vivo*. *Cell* **22:** 747-755

MacLeod, A.R., J. Karn, and S. Brenner. 1981. Molecular analysis of the *unc-54* myosin heavy chain gene of *C. elegans*. *Nature* **291:** 386-390

Maruyama, I.N., D.M. Miller, and S. Brenner. 1989. Myosin heavy chain gene amplification as a suppressor mutation in *Caenorhabditis elegans*. *Molecular General Genetics* **219:** 113-118

McLachlan, A.D. 1084. Structural implications of the myosin amino acid sequence. *Annual Review of Biophysics and Bioengineering* **13:** 167-189

Mello, C.C., J.M Kramer, D. Stinchcomb, and V. Ambros. 1991. Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO Journal*. **10**: 3959-3970.

Miller, D.M., F.E. Stockdale, and J. Karn. 1986. Immunological identification of the genes encoding the four myosin heavy chain isoforms of *Caenorhabditis elegans*. *PNAS* **83:** 2305-2309

Miller, D.M., I. Ortiz, G.C. Berliner, and H.F Epstein. 1983. Differential localization of two myosins within the nematode thick filaments. *Cell* **34:** 477-490

Moerman, D.G., and A. Fire. 1997. Muscle: structure, function and development. In "*C. elegans* II". Riddle, D.L., Dlumenthal, T., Meyer, B.J. and Priess, J.R., editors. Cold Spring Harbor New York, Cold Spring Harbor Press. 417-470

Moerman, D.G., S. Plurad, R.H. Waterston, and D.L. Baillie. 1982. Mutations in the *unc-54* myosin heavy chain gene of *Caenorhabditis elegans* that alter contractility but not muscle structure. *Cell* **29:** 773-781

Murata, S., T. Chiba, and K. Tanaka. 2003. CHIP: a quality-control E3 ligase collaborating with molecular chaperones. *International Journal of Biochemistry and Cell Biology* **35:** 572-578

Nasevicious, A. and S.C. Ekker. 2000. Effective targeted gene knockdown in zebrafish. *Nature Genetics* **26:** 216-220

Naylor, D.J. and F.U. Hartl. 2001. Contribution of molecular chaperones to protein folding in the cytoplasm of prokaryotic and eukaryotic cells. *Biochemical Society Symposium* **68:** 45-68

Nonet, M.L, J.E. Staunton, M.P. Kilgard, T. Fergestad, E. Hartwieg, H.R. Horvitz, E.M Jorgensen, and B.J. Meyer. 1997. *Caenorhabditis elegans rab-3* mutant synapses exhibit impaired function and are partially depleted of vesicles. *Journal of Neurosceince* **17:** 8061-8073

Obermann, W.H.J., H. Sondermann, A.A. Russo, N.P. Pavletich, and F.U. Hartl 1998. In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis. *Journal of Cell Biology* **143:** 901-910

Oldfors, A. H. Tajsharghi, N. Darin, and C. Lindberg. 2004. Myopathies associated with myosin heavy chain mutations. *Acta Myology* **23:** 90-96

Panaretou, B., C. Prodromou, S.M. Roe, R. O'Brian, J.E. Ladbury, P.W. Piper, and L.H. Pearl. 1998. ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone *in vivo*. *EMBO J* **17:** 4829-4836

Picard, D., B. Khursheed, M.J. Garabedian, M.G. Fortin, S. Lindquist, and K.R. Yamamoto. 1990. Reduced levels of hsp90 compromise steroid receptor action in vivo. *Nature* **348:** 166-168

Price, M. G., M.L. Landsverk, J.M. Barral and H.F. Epstein. 2002. Two mammalian UNC-45 isoforms are related to distinct cytoskeletal and muscle-specific functions. *Journal of Cell Science* **115**: 4013-4023.

Prodromou, C., S.M. Roe, R. O'Brian, J.E. Ladbury, P.W. Piper, and L.H. Pearl. 1997. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* **90:** 65-75

Rayment, I. 1996. Kinesin and myosin: molecular motors with similar engines. *Structure* **4:** 501-504

Riddle, D.L. and S. Brenner. 1978. Indirect suppression in *Caenorhabditis elegans. Genetics* **89:** 299-314

Riddle, D.L., T. Blumenthal, B.J. Meyer, and J.R. Priess. (Editors), 1997. *The Nematode Caenorhabditis elegans.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Scherrer, L.C., F.C. Dalman, E. Massa, S. Meshinchi, and W.B Pratt. 1990. Structural and functional reconstitution of the glucocorticoid receptor-hsp90 complex. *Journal of Biological Chemistry* **265:** 21397-21400

Scheufler, C., A. Brinker, G. Bourenkov, S. Pegoraro, L. Moroder, H. Bartunik, F.U. Hartl, and I. Moarefi. 2000. Structure of the TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* **101:** 199-210

Schmid MF, HF Epstein. 1998. Muscle thick filaments are rigid coupled tubules, not flexible ropes. *Cell Motility and Cytoskeleton* **41:** 195-201

Sellers, J. 2000. Myosins: a diverse superfamily. *Biochimica et Biophysica Acta* **1496:** 3-22

Shelton, C.A., J.C. Cater, G.C. Ellis, and B. Bowerman. 1999. The non-muscle myosin regulatory light chain gene *mlc-4* is required for cytokinesis, anterior-posterior polarity, and body morphology during *Caenorhabditis elegans* embryogenesis. *Journal of Cell Biology* **146:** 439-451

Smith, D.F. 1993. Dynamics of heat shock protein 90-progesterone receptor binding and the disactivation loop model for steroid receptor complexes. *Molecular Endocrinology* **7:** 1418-1429

Srikakulam, R. and D.A. Winkelmann. 2003. Chaperone-mediated folding and assembly of myosin in striated muscle. *Journal of Cell Science* **117:** 641-652

Titus, M. 1997. Unconventional myosins: new frontiers in actin-based motors. *Trends in Cell Biology* **7:** 119-123

Toi, H., K. Fujimura-Kamada, K. Irie, Y. Yakai, S. Todo, and K. Tanaka. 2003. She4p/Dim1p interacts with the motor domain of unconventional myosins in the budding yeast, *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **14:** 2237-2249

Vale, R.D. 1996. Switches, latches, and amplifiers: common themes of G proteins and molecular motors. *Journal of Cell Biology* **135:** 291-302

Venolia, L, and R.H. Waterston. 1990. The *unc-45* gene of *C. elegans* is an essential muscle-affecting gene with maternal expression. *Genetics* **126:** 345-354

Venolia, L., W. Ao, S. Kim, C. Kim and D. Pilgrim, 1999. unc-45 gene of *Caenorhabditis elegans* encodes a muscle-specific tetratricopeptide repeat-containing protein. *Cell motility and Cytoskeleton* **42**: 163-177.

Waterston, R.H., H.F. Brenner, and S. Brenner. 1974. Paramyosin of *C. elegans*. *Journal of Molecular Biology* **90:** 285-290

Waterston, R.H., R.M Fishpool, and S. Brenner. 1977. Mutations affecting paramyosin in *C. elegans*. *Journal of Molecular Biology* **177:** 679-697

Waterston, R.H., J.N Thomson, and S. Brenner. 1980. Mutants with altered muscle structure in *C. elegans*. *Development Biology* **77:** 271-302

Waterston, R.H. 1988. Muscle. In "The nematode *Caenorhabditis elegans*". W.B. Wood, editor. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 281-335

Waterston, R.H. 1989. The minor myosin heavy chain, mhcA, of *Caenorhabditis elegans* is necessary for the initiation of thick filament assembly. *EMBO J* **8:** 3429-3436

Wendland, B., J.M. Mccaffery, Q. Xiao, and S.D. Emr. 1996. A novel fluorescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homolog of mammalian eps15. *Journal of Cell Biology* **135:** 1485-1500

Wesche, S., M. Arnold, and R.P Jansen. 2003. The UCS domain protein She4p binds to myosin motor domains and is essential for class I and V myosin function. *Current Biology* **13:** 715-724

White, G.E., C.M Petry, and F. Schachat. 2003. The pathway of myofibrillogenesis determines the interrelationship between myosin and paramyosin synthesis in *Caenorhabditis elegans*. *Journal of Experimental Biology* **206:** 1899-1906

Williams, B.D. and R.H. Waterston. 1994. Genes critical for muscle development and function in *C. elegans* identified through lethal mutations. *Journal of Cell Biology* **124:** 475-490

Wong, K.C.Y., N.I. Naqvi, Y. Iino, M. Yamamoto, and M. Balasubramanian. Fission yeast Rng3p: an UCS-domain protein that mediates myosin II assembly during cytokinesis. *Journal of Cell Science* **113:** 2421-2432

Worm Atlas. http://www.wormatlas.org/handbook/mesodermal.htm/musclepartII.htm

Xu, Y. and S. Lindquist. 1993. Heat-shock protein hsp90 governs the activity of pp60v-src kinase. *PNAS* **90:** 7074-7078

Young,J.C., F.U. Hartl. 2000. Polypeptide release by Hsp90 involves ATP hydrolysis and is enhanced by the co-chaperone p23. *EMBO J* **19:** 5930-5940

Young, J.C., I. Moarefi,, and F.U. Hartl. 2001. Hsp90: a specialized but essential protein-folding tool. *Journal of Cell Biology* **154:** 267-273

Yu, Qin, and S.I. Bernstein. 2003. UCS proteins: managing the myosin motor. *Current Biology* **13:** R525-R527

Zhang, J.M., L. Chen, M. Krause, A. Fire, and B.M. Paterson. 1999. Evolutionary conservation of MyoD function and differential utilization of E proteins. *Developmental Biology* **208:** 465-472